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13. ABSTRACT ***(Maximum 200 Words)***

To assess, maintain, or improve a soldier's physical/physiological/psychological capability to function effectively under environmental and operational stress and to minimize adverse effects of stress on health safety and performance, the PBRC performs the following eight research tasks: 1) Clinical Laboratory for Human and Food Samples performs laboratory analysis of samples from studies conducted by the U.S. Army Research Institute of Environmental Medicine (USARIEM) and at PBRC in Tasks 4 and 8. 2) Stable Isotope Laboratory performs analyses to measure the energy expenditure and body composition of soldiers during prolonged field exercise and at PBRC in Tasks 4 and 8. 3) Stress, Nutrition and Mental Performance Laboratory continues multidisciplinary basic research studies of the interactions of stressors and nutrition on mental performance parameters in an animal model. 4) Stress, Nutrition and Work Performance uses human subjects to develop nutritional strategies to improve physical performance under intense physical stress. 5) Nutrient Database Integration Laboratory supports the Military Nutrition Division and PBRC research studies by providing dietary intake and analysis support. 6) Enhancing Military Diets targets health promotion through improved nutrition in basic combat training. 7) Stress Nutrition and Immune Function Laboratory has been inactive in this year (since 6/30/00). 8) Metabolic Unit Project allows new inpatient protocols to address specific issues in nutrition and metabolism that are important to the military mission.

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ANNUAL REPORT
US ARMY GRANT #DAMD 17-97-2-7013
July 1, 2000 – June 30, 2001

Introduction

On April 1, 1997, Grant #DAMD 17-97-2-7013, **Military Nutrition Research: Eight Tasks to Address Medical Factors Limiting Soldier Effectiveness**, was awarded to the Pennington Biomedical Research Center (PBRC) to address the following hypothesis: Soldier effectiveness in conditions of environmental, physiological and psychological stress can be enhanced by nutritional measures. The overarching rationale of the project is that better understanding of the nutritionally related mechanisms induced by stress will allow countermeasures to be designed and tested.

The goal of this research is to assess, maintain, or improve a soldier's physical/physiological/psychological capability to function effectively under environmental and operational stress and to minimize adverse effects of stress on health, safety and performance.

Technical Objective

This research continues the research relationship between PBRC and USARIEM over a five-year period. Those research relationships were established under prior cooperative agreements, #DAMD 17-88-Z-8023, "The Effect of Food, Diet and Nutrition on Military Readiness and Preparedness of Military Personnel and Dependents in a Peace Time Environment," and #DAMD 17-92-V-2009, "Military Nutrition Research: Six Tasks to Address Medical Factors Limiting Soldier Effectiveness."

The project allows for the continuation of the Clinical Laboratory for Human and Food Samples, Stable Isotope Laboratory, Enhancing Military Diets Project, and Nutritional Neuroscience Laboratory, all of which were initiated under Grant #DAMD 17-88-Z-8023. The project also expands the scope of research to allow for studies in humans of stress, nutrition and work performance, and for studies in humans and animals to evaluate the relationship of stress, nutrition and immune function. The grant provides a nutrient database laboratory. The project also allows for the utilization of PBRC's inpatient metabolic unit for a study designed by USARIEM investigators as detailed in the Metabolic Unit Project section.

Military Significance and Relevance to USARIEM Needs

The Stable Isotope and Clinical Laboratory methodologies are critical components of in-house military nutrition research of the U.S. Army Research Institute of Environmental Medicine. These extramural projects provide critical capabilities that do not exist in house, but are needed to fulfill the Army Surgeon General's responsibility to

provide nutritional research support to the DOD and Nutrition RDT&E Program.

The Nutritional Neuroscience Laboratory expands our knowledge of the effects of stress and the mechanisms of stress-related performance decrements and explores the ameliorative effects and mechanisms of action of dietary-induced alterations in behavior and cognitive function. Advances in this knowledge are the basis for developing safe and effective nutritional strategies to sustain and enhance soldier performance under conditions of environmental or operational stress.

The Enhancing Military Diets Project fulfills military needs to promote health, maintain readiness and sustain soldier performance. The Nutrient Database Integration Laboratory supports USARIEM projects assessing food intake in the field.

The Metabolic Unit Project provides the opportunity for an inpatient site for performance of specialized research utilizing the body composition assessment, energy expenditure assessment, metabolic kitchen services, and clinical laboratory expertise of PBRC. This project makes the unit available for USARIEM-sponsored studies and provides core support for Task 4.

This annual report describes progress during the fourth year of the grant. Discussions of the eight individual tasks funded under this grant follow.

TASK 1 – CLINICAL LABORATORY FOR HUMAN AND FOOD SAMPLES

I. Introduction

The Clinical Research Laboratory and the Food Analysis Laboratory continue to serve as reference laboratories for USARIEM investigators and research investigators at PBRC whose projects are funded by this grant. The laboratories provide expertise in routine and esoteric clinical testing, assistance with blood collection and processing for field studies, routine food analysis, and assistance with publications. Another service provided is method development, with a focus on automation. We continue to work closely with investigators at USARIEM to determine testing protocols and to provide timely reporting of results once assays are completed. The laboratory maintains high standards with regard to quality assurance and is certified by the College of American Pathologists and participates in the lipid standardization program sponsored by the Centers for Disease Control. During this year we provided analytical services for seven studies and provided field assistance with the Marine Women's Study at Parris Island, SC.

Priorities for the laboratory included method development and improved turn around time with reporting of results. In order to accomplish these goals, administrative restructuring occurred and Dr. Jennifer Rood was appointed as task leader.

New methods developed and implemented during this year include the following: fructosamine, hemoglobin A1C, ferric reducing antioxidant power (FRAP), retinol, lycopene, beta carotene, and alpha tocopherol. The assay for fructosamine was originally a manual method, but was adapted to run on the Beckman Synchron CX5. This adaptation to the automated chemistry instrument allowed increased throughput and better efficiency. The FRAP assay was set up and modified to run on the Beckman Synchron CX5. The FRAP assay is designed to measure the antioxidant capacity of a sample. The assay for hemoglobin A1C was set up on the Beckman Synchron CX7 which also facilitates high throughput. Both the CX5 and CX7 accept barcoded samples and are interfaced to the laboratory information system, eliminating many "hands on" steps. An HPLC assay was set up, validated, and implemented for the analysis of retinol, lycopene, beta carotene, and alpha tocopherol. In a single 35 minute run, all compounds are resolved and data is generated. Two detectors are used in sequence: beta carotene and lycopene use the UV/VIS detector and retinol and alpha tocopherol are detected using a fluorescence detector which provides an improved sensitivity over UV/VIS detection. Validation of the DPC Immulite 2000 was completed and this instrument was put into routine use. Tests offered on this high throughput immunoassay instrument include cortisol, C-reactive protein, DHEA-S, FSH, free T3, growth hormone, LH, progesterone, T4, testosterone, estradiol, T3, and TSH.

Testing was completed for six studies for USARIEM investigators this year and details of each project are described below. A total of 18,190 assays were completed for these projects.

II. Body

For the project entitled "The Effects of Modified Egg Protein and Antioxidants on Muscle Soreness and Strength after Eccentric Exercise" (EES), 15,674 assays were completed. The principal investigator of this trial was Dr. Jennifer Jenner who was a NSC Post-doctoral fellow at USARIEM. This study examined the effects of 1) both vitamins C and E, 2) immune egg protein, and 3) a combination of antioxidants and immune egg protein on muscle strength after eccentric exercise. Forty research volunteers consumed supplemented HooAH bars for 30 days prior to a bout of eccentric cycle exercise. Volunteers were monitored for seven days after exercise to determine muscle soreness, recovery and performance. It was hypothesized that the volunteers who consumed the bars containing either antioxidants, the immune egg protein or combination of protein and vitamins would experience less loss of muscle strength from eccentric exercise. Biochemical markers of muscle injury (plasma creatine kinase, myoglobin, lactate dehydrogenase) were measured, as were cytokines involved in the inflammatory process: interleukin (IL) -6, tumor necrosis factor (TNF)-alpha, and IL-1 beta. Additional tests performed included albumin, alkaline phosphatase, ALT, amylase, AST, bilirubin, BUN, calcium, cholesterol, chloride, CO2, creatinine, c-reactive protein, iron, ferric reducing antioxidant power, GGT, glucose, HDL cholesterol, potassium, LDL cholesterol, magnesium, sodium, prealbumin, phosphorus, total protein, triglycerides,

uric acid, and vitamin C. All of the tests were completed, results compiled, and data sent to USARIEM. Many values were less than the detectable limit for IL-6, IL-1B, and TNF-alpha. New "high sensitivity" methods for these analytes are being set up and samples from the EES study will be reanalyzed.

Analyses for the study entitled, "Assessment of Nutritional Status and Energy Expenditures and Determination of Gender Differences in Dietary Intakes of Combat Support Hospital Personnel Subsisting on Meal-Focused Versions of the Meal, Ready to Eat" (CASH), were completed. This study was a combined effort of the Military Nutrition Division, the Sustainability Directorate and the Science and Technology Directorate of the Soldier Systems Center, and PBRC to assess the nutritional adequacy for women of the Meal, Ready-to-Eat ration during a field training exercise. The study occurred during a field training exercise of a combat support hospital, and investigated gender differences in food selection, nutrient intake, and energy expenditure. A secondary purpose of the study was to assess the effects of a meal-focused concept of the Meal, Ready-to-Eat (MRE) on food selection, nutrient intake, and food wastage. The study also assessed the dietary habits and biochemical markers of nutritional status of male and female CSH personnel when in garrison prior to deployment, i.e., their nutritional readiness. A comprehensive list of biochemical tests was ordered and the last four tests were completed this year. Tests completed this year included retinol, beta carotene, alpha tocopherol, and lycopene. Results were compiled and the data sent to USARIEM.

Testing was completed for the study entitled, "Effects of Repeated Dosings of Caffeine on Vigilance, Cognitive and Physical Performance, and a Sentry Duty Marksmanship Task". The principal investigator was Harris R. Lieberman, Ph.D., an investigator at USARIEM. In this study, the effects of repeated administration of caffeine within a 12-hour test period were examined after one night of sleep deprivation. Caffeine, in the form of a 200 mg pill, was administered to volunteers at either three, four or six hours, over the course of six hours. Another group of volunteers were administered a carbohydrate based bar every four hours, containing 100 mg of caffeine, a dose equivalent to approximately one cup of coffee. Caffeine administration has previously been shown to have beneficial effects on performance in tasks that rely heavily on vigilance, and this study is intended to determine an optimal form of, and dosing schedule for caffeine administration. Volunteers were assigned into five groups: one placebo control group and four experimental groups comprised of up to 10 subjects each. Subjects were soldiers recruited from the Natick test subject pool or civilian volunteers recruited locally. Test measures were performed for caffeine and salivary melatonin. Results were compiled and sent to USARIEM.

Analyses were completed for the study entitled "Longitudinal Analysis of Bone Density and Stress Fracture Rates in a Population of West Point Graduates." This protocol was a follow-up to a study performed at the United States Military Academy, which evaluated bone health and stress fracture rates in a cohort of 262 male and

female cadets followed from 1990-1994. The current study brought back 121 of these graduates and repeated measurements of bone health, to include bone density, and other procedures employed in the first study approximately 10 years after the initial evaluation. Evaluation of these graduates at the 10 year mark was of particular interest for the 11 women and 13 men who were noted in the original study to not show the same rate of bone density increase with training as did the rest of the cadets studied. These individuals could be described as having a relative osteoporosis despite the loading of bone during exercise, which normally increases bone density. Tests for 25 hydroxy vitamin D and 1,25 dihydroxyvitamin D were completed, results compiled and data sent to USARIEM.

Analyses were completed for the study entitled, "Physical Fitness and Body Composition before and after a 2,000 km Unsupported Ski Trek across the Arctic Ocean" (Norwegian Army Arctic Study). The principal investigator was Marilyn Sharp from the Military Performance Division at USARIEM. This study followed physical and physiological responses to a 120-day, 2,000 km unsupported ski trek across the Arctic Ocean from Siberia to the North Pole and on to Ward Hunt Island In Northern Canada (AO2000 Expedition). Baseline testing took place at the U.S. Army Research Institute of Environmental Medicine in Natick, MA over a four-day period in the last week of January 2000, approximately three weeks prior to the start of the trek. Post expedition testing took place in mid-June and September 2000 at USARIEM. Biochemical measures of general health and nutritional status were conducted pre- and at two timepoints post-trek. Specific tests included glucose, BUN, creatinine, total protein, albumin, total bilirubin, lactate dehydrogenase, ALT, GGT, iron, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, vitamin C, RBC enzymes (EAST, ETK, and EGR), vitamin B12, cortisol, folate, ferritin, homocysteine, osteocalcin, testosterone, transferrin, and vitamin D. Results were compiled and data sent to USARIEM.

Analyses were completed for the study entitled, "Female Navy Energy Expenditure Study." The principal investigator was MAJ Beverly Patton from the Military Nutrition Division at USARIEM. This study assessed usual nutritional intake, nutritional status, and body composition of female sailors prior to six-month deployment. Fasting blood samples for select markers of nutrient and health status were collected from all subjects at the beginning of the study to assess the nutritional status of the subjects. Assays conducted included: retinol, retinol binding protein, serum carotenoids, serum total tocopherol, in vitro erythrocyte transketolase activation coefficient with thiamin pyrophosphate stimulation, in vitro glutathione reductase activation coefficient with FAD stimulation, in vitro aspartate aminotransferase activation coefficient with pyridoxal-5'-phosphate (PLP) stimulation, Vitamin C, 25-hydroxyvitamin D, folate, erythrocyte folate, homocysteine, B-12, osteocalcin, bone alkaline phosphatase, deoxypridinoline, ferritin, iron, erythrocyte protoporphyrin, transferrin saturation, mean corpuscular volume, total serum cholesterol, HDL cholesterol, serum triglycerides, apolipoproteins (A and B) and measures of general health including: alanine aminotransferase, albumin, bilirubin, BUN, creatinine, gamma glutamyl transferase,

glucose, lactate dehydogenase, and total protein. All analyses were completed, results compiled, and data sent to USARIEM.

The study entitled, "Assessment of Weight Status and Attrition of Female Marine Recruit during Recruit Training" is ongoing and data collection will continue through August, 2001. The principal investigator is LTC Gaston Bathalon from the Military Nutrition Division at USARIEM. This study will examine factors that may explain the observed weight gain and subsequent attrition of overweight female Marine recruits and seeks to identify factors that may explain why female recruits become overweight during recruit training and why overweight female recruits fail to lose weight during RT. This study will assess a large number of biochemical markers including total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, free fatty acids, fructosamine, hemoglobin A1C, cortisol, c-reactive protein, DHEA-S, FSH, growth hormone, leptin, LH, progesterone, free T3, T4, TSH, testosterone, neuropeptide y, corticotropin releasing factor, substance P, IL-1B, IL-6, and TNFa. Samples are being collected at four time points during the recruitment period (12 weeks). We are analyzing samples from approximately 60 recruits. Total analyses to be performed for this trial are 5,760. We are analyzing samples as they are collected and results are being sent to LTC Bathalon. Currently, 1,140 assays have been completed.

In addition to support of research initiated by USARIEM, the laboratory also provided service to investigators at PBRC who are funded by this grant. Specifically, the food analysis laboratory provided proximate analysis (fat, carbohydrate, moisture, ash and protein) on food samples prepared in our Metabolic Kitchen for the menu modification task.

III. Key Research Accomplishments

- Development of a new computer report program which improves turn around time for result reporting
- New method development/implementation for fructosamine, hemoglobin A1C, retinol, lycopene, beta carotene, alpha tocopherol, and FRAP
- Recertification by the College of American Pathologists
- Continued excellence in the Centers for Disease Control lipid standardization program
- Completion of testing for the EES study
- Completion of testing for the CASH study
- Completion of testing for the Caffeine and Vigilance study

- Completion of testing for the WestPoint study
- Completion of testing for the Norwegian Army Arctic study
- Completion of testing for the Female Navy-Energy Expenditure Study
- Routine testing begins on the DPC Immulite 2000

IV. Reportable Outcomes

Pending Funding

1. NIH R03. Diet Effects on Cigarette Smoke Induced Oxidative Stress. Submitted April, 2001. Jennifer Rood-Principal Investigator.

Presentations

1. Jennifer Rood, "Carotenoids and their role in cancer prevention and cancer treatment – an updated story." Roundtable, American Association for Clinical Chemistry, San Francisco, CA, July 2000.

2. Jennifer Rood, "Methods in Clinical Laboratory Medicine." Invited lecture, Louisiana State University, HUEC 2012, October, 2000.

3. Jennifer Rood, "Oxidative Stress Testing." Roundtable, American Association for Clinical Chemistry, Chicago, IL, July 2001.

4. Joanie Wilson, "The Relationship of Homocysteine and B Vitamins to Vascular Disease." Roundtable, American Association for Clinical Chemistry, San Francisco, CA, July 2000.

5. Joanie Wilson, "The Relationship of Homocysteine and B Vitamins to Vascular Disease." Roundtable, American Association for Clinical Chemistry, Chicago, IL July 2001.

Publications

1. Zachwieja JJ, Ezell DM, Cline AD, Ricketts JC, Vicknair PC, Schorle SM, Ryan DH. Short-term dietary energy restriction reduces lean body mass but not performance in physically active men and women. *Int J Sports Med* 22:310-316, 2001.

2. Waters WF, Magill RA, Bray GA, Volaufova J, Smith SR, Lieberman HR, Istre TA, Hurry M, Ryan DH. A randomized, double-blind, placebo-controlled clinical trial of tyrosine, phentermine, caffeine, and d-amphetamine comparing sleep parameters

during sleep deprivation and during recovery sleep. *Sleep* (submitted), 2001.

V. Conclusions

The clinical research laboratory and food analysis laboratory continue to provide valuable support services to enhance nutrition research in the military. The laboratory plays an important role in furthering the knowledge concerning nutrition in the military by providing routine and esoteric testing, custom method development, assistance with testing and collection protocols, field assistance with blood collection and processing, and collaboration on publications.

VI. References

None.

TASK 2 – STABLE ISOTOPE LABORATORY

I. Introduction

The research conducted by the Stable Isotope Laboratory is in the area of energy and water requirements, and changes in body water, of soldiers, often under harsh environmental conditions. The method used to determine energy requirements is the doubly labeled water (DLW) technique, which involves oral administration of water labeled with the stable isotopes, ^{2}H and ^{18}O. Saliva and urine samples are then obtained for periods of four to 14 days, longer with redosing. Water intake can be determined using only the ^{2}H labeled water. The use of doubly labeled water for measurement of energy expenditure was developed as a field technique for use in small animals (1). The method is based on the premise that after a loading dose of $^{2}H_2{}^{18}O$, ^{18}O is eliminated as CO_2 and water, while deuterium is eliminated from the body as water. The rate of CO_2 production, and, hence, energy expenditure, is calculated from the difference of the two elimination rates. The only requirement of subjects is to give urine and saliva specimens before and after drinking an initial dose of $^{2}H_2{}^{18}O$, and then return in one to two weeks to give a final urine specimen. During the period between the two urine and saliva samplings, subjects are free to carry out their normal activities and are not required to maintain extensive diaries. The doubly labeled water method has been extensively validated in humans under controlled settings (2), but there are confounding factors that need to be considered in field studies, particularly in Army Field Studies. Among these are change in location or food and water supply immediately preceding, or during an energy expenditure study. These changes may cause a change in baseline isotope abundance and, therefore, interfere with the accuracy of the energy expenditure measurement. This has occurred in a previous field training exercise involving the study of the MRE and RLW rations (3). This is a particular problem with studies such as the Ranger Training Studies (4), in which soldiers are moved to different parts of the country during the study.

Therefore, a group not receiving labeled water must be followed to make any corrections in baseline isotope shifts.

Hydration status is another main focus for some Army studies. Using the cheaper and more readily available deuterium tracer, either changes in total body water (5,6) can be followed during a study, or water turnover (intake) (7,8) can be measured during a study.

One advantage of the DLW method is that it uses stable isotopes so there is no radiation exposure. The method uses two heavy isotopes of water, which are naturally occurring in food and water. There are no known side effects of either isotope at the doses given in DLW studies and has been used extensively to study energy expenditure during pregnancy (10,11) lactating women (12), and infants for measurement of energy expenditure and human milk intake (13-15).

The Stable Isotope Lab was involved in several Army research projects during the current year. These are described below.

II. Body

Stable isotope studies were completed for two studies, completed for two out of three iterations of one, and a fourth study was begun. The first study employing doubly labeled water was a shipboard study, a collaborative effort with NHRC and USARIEM. Women (16) and men (9) were dosed with doubly labeled water for measurement of energy expenditure.

Analyses for samples from a shipboard study conducted with NHRC and USARIEM as part of Dr. DeLany's DWHRP study of energy requirements in female, compared to male military personnel, have been completed. Total body water (TBW) and fat free mass (FFM) were similar regardless of the isotope, $^{2}H_2O$ or $H_2{}^{18}O$, from which it was calculated (Table 1). The females had significantly less TBW and FFM than the males. Total energy expenditure (TEE) was calculated using a 2 point method and by linear regression of the sample points collected on day 0, 2, 7 and 8. There was no significant difference in TEE regardless of the method by which it was calculated (Table 1). The females expended significantly fewer calories than their male counterparts. There was a significant correlation between total body water and total energy expenditure; the greater the FFM, the more total energy expended (TEE = 1057.9 + 37.4*FFM; r^2=0.62).

Table 1. Stable Isotope Data

Subject Number	Total Body Water (kg)		Fat Free Mass (kg)		Energy Expenditure (kcal/d)	
	O^{18}	Deuterium	O^{18}	Deuterium	2 Point Method	Regression Method
Females:						
Average	33.1	33.3	45.2	45.5	2744	2808
St Dev.	5.4	5.2	7.4	7.2	438	429
Males:						
Average	49.4	49.7	67.4	67.9	3419	3473
St. Dev.	8.7	7.9	11.9	10.8	808	807

Isotope analyses for the Ft. Carson study of energy balance with the 10th Special Forces Group (SFG) were completed. Nineteen subjects were dosed with doubly labeled water and three undosed subjects served a placebo subjects to examine any shifts in baseline isotope abundance. There were nine Special Forces Qualified (SFQ, elite soldiers who must be ready for deployment at all times) and 10 Support (SPT) personnel. Total daily energy expenditure (TDEE) of the SPQ group was 4099 ± 740 kcal/d, while that in the SPT group was 3361 ± 939 kcal/d. There was no difference in energy intake between the two groups. The SPQ group had a 1123 ± 1017 kcal/d deficit, while the SPT group actually had a 122 kcal/d energy surplus.

A Sustained Operations Study was initiated by USARIEM to characterize cognitive and physiological changes in soldiers participating in sustained operations. One aspect of this study was to examine water status using stable isotopes to study total body water and water turnover. For the first two iterations of the Sustained Operations Study, 12 subjects were dosed with deuterated water to study TBW and water turnover, while two subjects who were not dosed served to examine any changes in naturally occurring isotope abundance. In a third iteration, an additional four subjects were dosed along with two undosed subjects. Isotope analyses, including repeats, and calculations were completed for the first 12 subjects.

Protocol discussions with Reed Hoyt regarding the Warfighter Physiological Status Monitoring (WPSM): Thermal Status And Water Intake During A Warm Weather U.S. Marine Corps Marksmanship Training Course Study were conducted. Thermal status and water intake were assessed in 27 USMC volunteers (24 males, three females; average age = 25; ht = 177.24cm; body wt = 77.7 kg; load wt = 26.8 kg) over four days during a marksmanship training course at The Basic School, Quantico, Virginia. Total water turnover will be measured by the deuterated water eliminated method; while wearable sensors were used to collect body core temperature (telemetry

pill), heart rate (HR), activity (sleep/wake) patterns, metabolic cost of locomotion (pedometry) and water consumption (instrumented bladder-type canteen). Water intake from the instrumented bladder canteen during the training day ranged from about 1 to 3 L (mean = ~2 liters) during the temperate training day, and about 1.2 to 6.6 L (mean = ~2.5 L) on the hot weather day. Samples for isotope analyses from this study should be received early next quarter.

III. Key Research Accomplishments

- During the Shipboard study, the average daily energy expenditure of the female subjects was 2808 $\pm$ 429 kcal/day. This is significantly less than the energy expenditure of the male subjects. However, this difference in daily caloric energy expenditure can be explained by a difference in fat free mass. The men had a significantly greater fat free mass than the women.

- A group of Special Forces Qualified soldiers were shown to expend 4100 kcal/d, while consuming only 3000 kcal/d, leading to a 1,100 kcal/d deficit. A group of support personnel studied at the same time expended 3360 kcal/d and had a slight energy surplus of 120 kcal/d.

- Isotope analyses for two iterations of a Sustained Operations Study of water balance were completed, while samples for the final iteration have been processed and are ready for analyses.

- Protocol development for a Warfighter Physiological Status Monitoring Study were completed and samples will arrive next quarter.

IV. Reportable Outcomes

Publications

1. Tharion WJ, DeLany JP, Baker-Fulco CJ. Total daily energy expenditure of male and female soldiers during a field training exercise. *FASEB J* 15:A988, 2001.

V. Conclusions

The average daily energy expenditure of the female shipboard subjects was 2808 $\pm$ 429 kcal/day. This is significantly less than the energy expenditure of the male subjects. However, this difference in daily caloric energy expenditure can be explained by a difference in fat free mass. The men had a significantly greater fat free mass than the women.

Special Forces Qualified soldiers were shown to have a high energy expenditure of 4100 kcal/d, with a subsequent energy deficit of 1100 kcal/d.

This task produces research results which help in defining operational rations for servicemen and servicewomen.

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TASK 3 – STRESS, NUTRITION AND MENTAL PERFORMANCE

I. Introduction

The objective of the research conducted in this Task is to identify the physiological and biochemical mechanisms underlying behavioral responses to stress using rodent models. Once these mechanisms have been identified the goal is to develop appropriate nutritional strategies to reduce, or prevent, negative behavioral and biological responses to stress. Successful interventions for ameliorating the negative aspects of stress in animal models can be transferred to the clinical setting to determine their efficacy in human subjects.

Achieving an ideal body weight and composition is essential for optimal performance and for Army personnel to continue their valuable service to the United States. Therefore, it is essential to identify strategies that allow Army personnel to maintain their ideal body weight while also retaining optimal physical performance. The rodent is used as a model to gain new basic information on the neurochemical and physiological mechanisms of weight gain and loss. This information will be used to aid the design of novel approaches for control of body weight and fitness of Army personnel.

II. Body

This task has undergone significant changes during the current year of funding, due to the change in leadership of the Task. Dr. Roy Martin has assumed the role of task leader. Some of the projects that were being conducted at PBRC are now being performed at University of Georgia in the laboratory of Ruth Harris. Other projects

were ended when key personnel left, these include the investigation of the effects of stress on immune function, the effect of stress on brain insulin action and cognitive function, and the effect of changing diet composition on the behavior responses of sleep deprived rats.

The projects that are being continued are the chronic effects of repeated acute stress on body weight regulation and identification of genetic markers for stress responsiveness. We have completed a number of experiments investigating physiological, neurological and behavioral responses in rats that have been exposed to repeated restraint and then subjected to a second stressor after a period of recovery from the restraint. Repeated restraint causes a chronic down-regulation of body weight that lasts for at least 3 months after the end of restraint (see Appendix). Previous experiments have not been able to demonstrate a chronic disruption of CRF or catecholamine pathways that are known to be involved in mediating the stress response (3) although blockade of CRF receptors during restraint prevents weight loss (4). The weight loss is primarily due to a deficit in energy intake during the period of restraint that is not compensated for once stress has ended (2) as there is no measurable difference in energy expenditure of the animals other than during the time of restraint (see Appendix). Recently we have been trying to determine whether rats that have been exposed to repeated restraint are an animal model of post-traumatic stress disorder. Pilot data from a study in which restrained rats were subsequently exposed to the severe stress of restraint in ice-cold water suggested that there was an increased endocrine response to the second stressor in rats that previously been exposed to repeated restraint, compared with those that had not been stressed previously (see Appendix). Subsequent studies measuring behavior and endocrine responses to a second, mild stress in repeatedly restrained rats did not show any difference in the stress responses of the rats (see Appendix), however, a recently completed study shows a different endocrine response to a 30 minute restraint stress in rats 84 days after they had been subjected to repeated restraint (see Appendix). Therefore, we can conclude that exposure to an acute stress causes prolonged changes in body weight regulation of rats and also modifies their responses to subsequent stressors. Future experiments will define the most appropriate model to use in the investigation of whether repeated restraint stress induces post-traumatic stress disorder. If we establish the appropriate model then we will investigate the neurological and biochemical mechanisms responsible for the response.

In the second project we have been investigating whether activity of the melanocortin system can be considered a genetic marker for stress responsiveness. We found that mice that over-express agouti protein show an exaggerated endocrine, behavioral and catecholamine response to stress (6). Agouti protein is an endogenous antagonist of melanocortin receptors which have been implicated in the regulation of food intake, immune function, feedback regulation of the CRF system and activation of the adrenal gland. In mice, agouti expression is normally limited to the brain and to skin and hair, where it regulates pigmentation and coat color (1). In humans, agouti

protein is also expressed in adipose tissue (5) which makes it a realistic protein to use as a marker as it would be possible to obtain tissue samples from soldiers.

In order to confirm these results we hoped to test the effect of acute inhibition of melanocortin receptors on the stress response. It was not possible to buy large quantities of the antagonist, therefore, we treated mice with α-MSH, which activates melanocortin receptors. Theoretically, this should have minimized the stress response of the mice and did inhibit corticosterone release but had no effect on the behavior of the animals (see Appendix). We still intend to determine whether acute antagonism of central melanocortin receptors influences the stress response in rats, but it is possible that the change in sensitivity is only present in transgenic mice that have developed with an abnormal melanocortin system. Because the objective of this project is to identify genes that can be used as markers for stress responsiveness it is appropriate to use information from transgenic animals in which the level of expression of specific genes has been modified.

Starting January 1, 2001 the Task III staff at PBRC included Dr. Jun Zhou (100%), Xiaochun (100%) and Dr. Roy Martin (20%). By the beginning of June, Dr. Martin's time had increase to 33%, and two more members joined the team (Bing Li and Colby Danna). During the first three months Dr. Martin made weekly conference calls to the staff at PBRC, monthly trips to Baton Rouge and exchanged email messages to the staff at PBRC organizing interviews for bringing the new team of scientists on board in a timely manner. The hiring plan for Task Three was developed and approved. A five-year research plan developed and presented to the Army review panel and two abstracts to the neuroscience meeting were submitted.

We propose that the initiation of meals and termination of meals are partially controlled by how the brain senses glucose availability. In addition, we propose that specialized brain cells have similar gene expression as in the pancreatic beta cells. We have found that streptozotocin a specific toxin for pancreatic beta cells will alter the brain's ability to alter feeding response to hypo- or hyper-glycemic conditions (7). In addition, intracerebroventricular administration of antisense oligodeoxynucleotide against GLUT2 glucose transporter mRNA reduces food intake, body weight and glucoprivic feeding response in rats (8). Therefore, we have developed a plan to examine the expression of these beta cell genes in the hypothalamus and hindbrain during hunger and satiety. The first step in this plan was the cloning of glucokinase, glucose transporter-2, pre-proglucagon, glutamate decarboxylase, leptin and glucagon-like-peptide-1 receptors. The processes involved were amplification partial cDNAs by RT-PCR and cloning of partial cDNAs into pCR II. These clones that contain partial cDNA of the genes found in the beta cells of the pancreas will be used in the study of the brain's sensing of glucose as a signal of short-term control of feeding behavior. Both leptin (9) and glucagon-like-peptide-1 (10) have been shown to reduce the feeding response to glucoprivation and therefore likely to be key factors in the modification of both short-term and long-term control of food intake controlled by glucose status.

Furthermore, localization of glucokinase mRNA in hypothalamic areas known to contain glucosensing neurons (11) support a role for "beta-like" cells that integrate multiple signals relating to energy balance and body weight control.

The second step in this plan was to develop methods to identify what cells of the brain express these genes. Since the expression of these genes is expected to be low in the brain only involving a small percentage of total brain cells, it was necessary to improve on the sensitivity of existing procedures. This was done by setting up a new method of double-color fluorescence *in situ* hybridization. Xiaochun combined procedures from two different methodologies to establish the novel approach. With this method we can detect with greater sensitivity multiple gene mRNA expression in the same tissue section. The double-color fluorescence *in situ* hybridization is described in the appendices and will be used in many future studies of other genes associated with hunger, satiety and food reward. Results so far are as follows: No signals for GLUT2 or GK mRNA were detected in arcuate nucleus. In hindbrain, GLUT2 and GK mRNA was found co-expressed in mlf (medial longitudinal fasciculus) area from Bregma –14.00mm to –10.00mm, and GK expression was much stronger than GLUT2 expression in the whole area. In area postrema and nucleus of solitary tract, GLUT2 was also detected in individual neuron cells, while GK was not co-expressed in these areas with GLUT2. Localization of glucokinase mRNA in hypothalamic areas known to contain glucosensing neurons (11) support a role for "beta-like" cells that integrate multiple signals relating to energy balance and body weight control.

Overeating has been linked to highly palatable, energy dense foods. Foods that have a high reward stimulus lead to over-consumption of foods and obesity. The second area of study has been the reward system and the overriding of normal satiety signals. The dopamine system is one of the major reward mechanisms associated with palatable foods (12). Therefore, studies were conducted on brain reward stimulus and the role of dopamine system in feeding behavior in both satiated and hungry animals. From our initial observations it appears that food induced reward will provide the necessary characteristics to test our hypothesis that the dopamine reward system acts differently in hunger and satiated states. The dopamine system of the substantia nigra and ventral tegmentum is activated in normal rats when they have access to food. This activation is increased while the rats have more palatable food, like cookies (13).

III. Key Research Accomplishments

- Identification of Agouti protein as a potential maker for stress-responsiveness
- Development of the model of repeated restraint stress as a potential model or Post-Traumatic Stress Disorder
- The double-color fluorescence *in situ* hybridization was developed for studies of genes associated with hunger, satiety and food reward

- Dopamine transport mRNA expression was increased in satiated rats when fed a palatable diet, but not in hungry rats induced by 2-DG injection

IV. Reportable Outcomes

Manuscripts

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V. Conclusions

A significant amount of work has been done on the model of repeated restraint which induces a chronic down-regulation of body weight. This unique model allows us to investigate the negative effects of a mixed stressor on energy balance. Elucidation of the mechanisms responsible for this response may allow development of a program that could either prevent or reverse the stress-induced suppression of appetite in soldiers during missions or training exercises that involve both physical and psychological stress. The loss of appetite exaggerates stress-associated weight loss and impairs physical performance. We are currently trying to establish whether, or not, this is an animal model for Post-Traumatic Stress Disorder. If this is established then it will provide an ideal system in which to test potential preventive, or corrective, treatments for this disorder.

We have also made significant progress in the investigation of genetic markers for stress-responsiveness. Evidence that mice that over-express agouti protein are more responsive to stress than their wild-type controls provides the first evidence for a new marker and also implicates the melanocortin system in the behavioral and physiological response to stress.

The tools and methods for investigation of beta-like cells in the brain are nearly in place. Early results of these studies suggest that we will be able to identify and characterize these brain cells. However, our initial trials have shown that glucose-sensing mechanisms of the brain are important in controlling meal initiation and termination of meals and that key components of the sensing mechanism is found in specific areas of the brain. This information will provide the focus necessary to study how metabolic signals are translated to feeding or satiety signals. These mechanistic studies are necessary to develop a strategic approach to altering feeding behavior in unique ways. One dietary approach to be tested next year is the use of foods that have a low glycemic load. The low glycemic index foods are ones that release glucose at a slow rate so that blood glucose levels are maintained for extended periods of time. It is

hypothesized that foods with a low glycemic index will produce a delay in hunger signals associated with hypoglycemia and reduce overall food intake and body weight.

Food reward and the overriding of satiety signals lead to obesity. The mechanisms appear to involve the dopamine system and specifically the dopamine transporter as a marker of dopamine stimulation. The palatability of food in this particular experiment is defined as the preference and amount of the food consumed. These basic food reward studies are aimed at identifying genes that control over-consumption of palatable foods. The long-range goal is to be able to control those genes in order to prevent overeating associated with food rewards mechanisms in the brain.

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TASK 4 – STRESS, NUTRITION AND WORK PERFORMANCE

I. Introduction

The overall objective of this task has been to conduct clinical research to evaluate the efficacy of nutritional strategies that could enhance physical performance under conditions of extreme physical stress and/or energy deficit. In past years, the group of Dr. Jeff Zachwieja, who used muscle endurance strength and anaerobic capacity as endpoints, conducted most of these studies. However, with the departure of Dr. Zachwieja, and the arrival of Dr. Eric Ravussin (June, 2000), this task has been refocused to now address two major research questions: a) continue studies of nutritional approaches to enhance performance, and b) initiate new studies designed to better understand the factors involved in the etiology of weight gain and obesity.

Achieving an ideal body weight and composition is absolutely essential for optimal performance, especially for Army personnel to continue their valuable service to the United States. It is common knowledge that most individuals who lose weight by conventional means (diet and exercise) will regain the weight within five years. Complex interactions of energy intake and expenditure and the partitioning of energy between muscle and adipose tissue compartments regulate body weight. Obviously, genetics, nutrition, stress and physical activity all influence energy balance and partitioning of energy. The increasing number of Army personnel not meeting body weight standards and the shrinking pool of adolescents to recruit from (lower natality in the 80s and increased prevalence of obesity) is of concern to the Department of Defense. New research studies need to be undertaken to better understand the factors leading to weight gain in adults and to prevent obesity at a younger age. Studies designed to improve our understanding of the interaction between environmental

factors (such as nutrition and physical activity) and the genetic predisposition for weight gain need to be performed.

In response to this challenge, Dr. Eric Ravussin decided to reorganize the Task around three major research themes: a) Metabolic Understanding of Energy Balance; b) Influence of Diet on Training and Performance; and c) Prevention of Obesity. Since weight gain and obesity are clearly the result of an interaction between environmental factors and genetic predisposition, a laboratory of functional genomics will support these three areas of research.

II. Body

Achieving an ideal body weight and body composition is essential for optimal performance especially for Army personnel. It is, therefore, imperative to better understand the interactive process between physiological functions, genetic makeup and environmental conditions that favors weight gain. Similarly, studies designed to identify the most appropriate dietary regimen for achieving peak performance are necessary. Such studies will be essential to identify strategies that will allow Army personnel to maintain ideal body weight, while also retaining optimal physical and cognitive performance. Because of the alarming increase in the prevalence of childhood obesity, it is now clear that the DOD will have increasing difficulty recruiting as the pool of young, fit individuals is shrinking on a yearly basis. Since the seeds of adult obesity are sown in childhood when behavioral patterns are set, and since childhood overweight strongly correlates with adult overweight, the new task will also have a laboratory investigating childhood obesity and its prevention.

The mission of this newly designed Task is, therefore, to conduct innovative clinical research designed at improving health and performance in people with relevance to military personnel. The Task leader, Dr. Eric Ravussin, is a renowned investigator in the field of energy balance, diabetes and physical activity. The new task is now structured around the following four sections: a) Metabolic Understanding of Energy Balance (deJonge and Ravussin); b) Influence of Dietary Fat on Training and Performance (Larson-Meyer and Ravussin); c) Obesity Prevention (Sothern and Ravussin) and d) Functional Genomics of Energy Balance and Training (Argyropoulos and Ravussin). The mission of this newly designed Task is, therefore, to conduct innovative clinical research designed at improving health and performance in people with relevance to military personnel. The Task leader, Dr. Eric Ravussin, is a renowned investigator in the field of energy balance, diabetes and physical activity. The new task is now structured around the following four sections: a) Metabolic Understanding of Energy Balance (deJonge and Ravussin); b) Influence of Dietary Fat on Training and Performance (Larson-Meyer and Ravussin); c) Obesity Prevention (Sothern and Ravussin) and d) Functional Genomics of Energy Balance and Training (Argyropoulos and Ravussin).

To develop the new research themes described above, Dr. Eric Ravussin has hired three key faculty position scientists.

- In September of 2000, Dr. Melinda Sothern was hired as an Assistant Professor to develop new programs of prevention of adolescent obesity. Dr. Sothern has a strong background in behavioral methods to impact obesity and the associated chronic complications using comprehensive physical activity and dietary guidance in a community-based setting involving schools and families.

- In January of 2001, Dr. D. Enette Larson-Meyer was hired to undertake the new studies of the interaction between diet composition and performance/trainability. Dr. Larson-Meyer has a strong background in both dietetics and exercise physiology and, more recently, has been involved in very innovative studies of the impact of intramyocellular fat measured by spectral nuclear magnetic resonance (sNMR).

- To support these areas of research at the molecular level, Dr. Ravussin has hired Dr. George Argyropoulos in February of 2001 to lead the new Laboratory of Functional Genomics. Dr. Argyropoulos has a very good training in genetics and, more recently, has been involved in the functional characterization of genes including the promoter and intronic areas of these genes.

Each of the projects is described separately below.

Project A - Metabolic Understanding of Energy Balance

In collaboration with Dr. Lilian deJonge, Dr. Eric Ravussin studies energy metabolism responses to dietary and exercise interventions. There is growing evidence that obesity is caused not only by chronic positive energy balance, but also by the lack of quick adaptation to acute perturbation in energy balance or dietary fat content. The project uses state-of-the-art methods, including respiratory chambers, metabolic carts and doubly labeled water to assess energy metabolism, a heart spectral analysis and microneurography to measure autonomic nervous system balance and microdialysis to determine local substrate and hormone release.

The first proposed study was designed to investigate the mechanisms underlying the inter-subject variability in energy metabolism in response to perturbation of energy balance. In this study, we wish to measure metabolic responses to acute overfeeding (three days) and complete starvation (three days) on 24-hour energy expenditure and macronutrient oxidation measured in a respiratory chamber in obesity prone (non-obese individuals with a family history of obesity and post-obese individuals) and obesity resistant (never obese without a family history of obesity) individuals. We also wish to determine the relationship between these metabolic responses and the activity of the autonomic nervous system. Finally, since these metabolic adaptations are taking place

primarily in skeletal muscle and adipose tissues, we will measure gene expression in these tissues in response to overfeeding and fasting.

Volunteers will be admitted to the inpatient unit of the PBRC on three separate occasions. On each visit, they will spend three days in the metabolic chamber while either fasting or receiving 100% or 200% of their initially estimated 24-hour energy expenditure. All metabolic chamber periods will be preceded by a three-day run-in period during which a weight maintaining diet containing 37% fat will be eaten. All individuals will participate in all three conditions starting with the isocaloric condition. The order of the two other tests will be randomized.

Project B – Influence of Dietary Fat on Training and Performance

The quantity of lipid droplets stored within skeletal muscle fibers intramuscular lipids (IML) may be important in controlling lipid utilization performance during exercise. Under normal dietary conditions, skeletal muscle contains significant stores of IML that serve as an important source of energy during exercise, particularly prolonged moderate-intensity exercise. Previous studies have demonstrated that IML are significantly depleted after moderate to strenuous endurance exercise such as those encountered in the Army personnel during prolonged strenuous endurance exercise. Studies have also alluded to the possibility that extremely low-fat diets (10-15% total energy from fat) may be detrimental to performance in endurance-trained individuals possibly by compromising IML stores.

While it is well-established from studies in male athletes, that a certain quantity of dietary carbohydrate (6-10 g/kg of body weight) is necessary to maintain adequate muscle glycogen stores and optimize performance, little is known concerning the quantity of dietary fat that will both optimize performance and promote good heath in active individuals such as military troops and highly-trained individuals.

Dr. Enette Larson-Meyer is, therefore, initiating two studies that form the basis for subsequent experiments.

- The influence of two diets, one low in fat and high in carbohydrate (20% fat, 65% carbohydrate, 15% protein) and one moderate in both fat and carbohydrate (35% fat, 50% carbohydrate, 15% protein) on the adaptation to an intense exercise training program in healthy, but previously inactive individuals.

- The influence of a controlled, very low-fat (10% fat) and moderate fat (30%) recovery diet on replenishment of IML and glycogen stores following a bout of prolonged, moderate exercise and subsequent performance in trained, endurance athletes.

In both studies, we will measure the influence of the diet/training interaction and the composition of the recovery diet on gene expression. The combination of these two studies will help determine the optimal dietary fat composition in two groups of subjects, a previously inactive group entering into an intense training program (similar to military basic training) and a highly active endurance trained group (similar to military special forces, Rangers, Seals, etc.).

Importantly, Dr. Enette Larson-Meyer will develop the technique of spectral NMR in Baton Rouge, which will be instrumental for future determination of intramyocellular lipid measured in a non-invasive way.

Project C – School/Community Intervention to Prevent Adult Obesity and Overweight Adolescence - A Three-Year Randomized and Controlled Trial

Americans are becoming obese earlier and the long-term physiological metabolic consequences, even if not fully understood, are deleterious. Therefore, research efforts should focus on methods and techniques that primarily halt the onset of obesity early in life, but moreover, prevent overweight teenagers, who currently number 30% of the adolescent population, from becoming obese young adults.

In this project, let by Dr. Melinda Sothern, we propose to examine the feasibility and efficacy of a three-year randomized and controlled trial of a community/school-based intervention program to prevent adult obesity in overweight adolescents (13-15 years of age). The intervention program will be implemented in adolescents in a family-based school/community setting. If this intervention proves to be feasible and effective, it could be implemented on a larger scale. We propose to adopt an enhanced version of the Committed to Kids program that we have used at PBRC and at the LSU Health Science Center in New Orleans for over eight years. The annual field measurements will include weight, height, body mass index, waist and hip circumferences, body composition using (BIA), blood pressure, physical fitness, family and medical questionnaire, self report Tanner Stage, and physical activity by questionnaires.

Project D - Functional Genomics of Energy Balance and Training

Two experimental approaches have been underway in the last five months (Dr. Argyropoulos joined the PBRC on January 24, 2001) and those projects deal with polymorphisms in the promoters of two candidate genes for food intake and energy balance: the Agouti Related Protein (*hAGRP*) and *Resistin*, respectively.

Experiment 1. A Role for the Agouti Related Protein in Food Intake and Human Obesity - George Argyropoulos

Introduction

Recently, a homolog of the murine agouti gene (*mAGOUTI*), termed Agouti Related Protein (*mAGRP*), was discovered in the arcuate nucleus of the hypothalamus with a 10-fold higher melanocortin antagonistic effect and was upregulated in obese and diabetic mice. *mAGRP* stimulates hyperphagia when administered intracerebroventricularly (i.c.v). Coexpression experiments have colocalized *mAGRP* in hypothalamic neurons overexpressing neuropeptide-Y (*NPY*) and have shown that *mAGRP* mRNA levels increase 18-fold in response to fasting. Ablation of the arcuate nucleus by neonatal administration of monosodium glutamate obliterates nearly all *mAGRP*-immunoreactivity in the murine hypothalamus. Transgenic mice overexpressing *mAGRP* exhibit severe obesity, increased body length, hyperinsulinemia, late-onset hyperglycemia, pancreatic islet hyperplasia, and reduced corticosterone level. Leptin has been shown to downregulate *AGRP* expression, while *hAGRP* can itself be a negative regulator of leptin action when administered to mice i.c.v. Two hours after 2-deoxy-D-glucose injection, food intake and *rAGRP* expression were increased in rats suggesting that arcuate nucleus neurons expressing NPY and AGRP are susceptible to glucose homeostasis. Central administration of ghrelin, a growth-hormone-releasing acylated peptide from the stomach that induces adiposity in rodents has been reported to increase *rAGRP* mRNA and food intake in rats.

A truncated variant of the human *AGRP* gene, *hAGRP*(87-132) containing the 46 carboxyl-terminus cysteine-rich residues, was found to be biologically active and equipotent to the mature mouse homolog. Moreover, the *hAGRP*(87-132) human variant was found to bind effectively to the melanocortin receptors MC3R, MC4R, and MC5R, and to inhibit binding of alpha-melanocyte stimulating hormone (α-MSH) to these receptors. In other experiments, chemically synthesized peptides of the amino-terminus of the human *AGRP* gene {*hAGRP*(25-51) or *hAGRP*(54-82)} were devoid of antagonistic action, while, the carboxyl-terminus peptide *hAGRP*(83-132) was potently active, as tested for anti-melanocortin activity using *Xenopus laevis* dermal melanophores.

Leptin reduction results in inactivation of the proopiomelanocortin hormone neurons in the arcuate nucleus, which translates into decreased production of α-MSH. In the paraventricular nucleus, increased amounts of NPY/AGRP block the action of α-MSH by antagonizing its receptor MC4R. This means that the anorectic properties of α-MSH are diminished which leads to an increase in satiety and the associated increase in food intake. If food intake is not reduced by a reversal of this cascade, perhaps through a signal that feeds back to the adipocytes to increase leptin production, the inevitable outcome may be hyperphagia and the development of obesity.

The scope of the present study was to determine the promoter of *hAGRP* and screen for the presence of genetic variants. We hypothesize that genetic variants in the promoter of *hAGRP* could affect the binding of transcription factors to recognition sites, thus, up- or down-regulating expression of the gene. We have determined upstream genomic sequences for the human *AGRP* as well as nucleotide sequences with significant promoter activity. Multiple cis-acting elements were identified including a non-canonical TATA-box, a CCAAT box, and a putative recognition site for the Signal Transducers and Activators of Transcription (STATs). A novel polymorphism was identified in the minimal promoter, –38C>T, and had significant impact on promoter activity and affinity to bind transcription factors. Finally, the *C/C* genotype of the polymorphic site was found at significantly higher frequencies in obese and type 2 diabetic Africans. The impact of the –38C>Tpolymorphism on the transcription of *hAGRP* and its potential role in the regulation of appetite in the low-fat African diets are discussed.

Materials and Methods

Amplification primers were designed according to the DNA sequence with GenBank Accession number U88063. To determine 5′ upstream sequences, the Genome Walker kit (CLONTECH, Palo Alto, CA) was used at conditions as specified by the manufacturer. The primers used to obtain the sequence were as follows: {*hARTR4*: 5′-gcctaatgaagggccacac-3′ and *hARTR5*: 5′-cacaggacacccacctagga-3′}. Sequencing was performed with an ABI 373 Sequencer (Applied Biosystems, Foster City, CA). TRANSFAC analyses were performed using the algorithm available at: [http://www.cbil.upenn.edu/tess/index.html].

Constructs to examine promoter activity were directionally cloned (with *Sac I* and *Xho I* restriction recognition sites incorporated into the primers) into the pGL3 luciferase basic vector (Promega, Madison, WI). The two polymorphic constructs were generated by amplification of genomic DNA from homozygous (*C/C* or *T/T*) individuals. Cell culture was carried out under standard conditions in a humidified incubator at 37 °C and 5% CO_2. The GT1-7 cells were the kind gift of Dr. Mellon and were grown in DMEM, L-glutamine, and 10% fetal calf serum (FCS) supplemented with the antibiotics penicillin-streptomycin-gentamycin. The CHO cells were purchased from the American Type Tissue Collection (Manassas, VA) and were grown in MEM Eagle 1X, Earls salts with L-glutamine, and 10% FCS supplemented with the same antibiotics. The cells were serum starved for 24 hours prior to transfection. Transient cotransfections with the constructs and β-gal plasmids were carried out for 24 hours in the absence of serum using the Geneporter2 transfection reagent as prescribed by the manufacturer (Gene Therapy Systems, San Diego, CA). Subsequently, the media were supplemented with 20% FCS for 24 hours. Cells were harvested using 1x Geneporter2 lysis buffer and the lysates were assayed for luciferase and β-galactosidase activities, as prescribed by the assay manufacturer (Promega, Madison, WI) in a luminometer (Zylux Corporation, Pforzheim, Germany). All luciferase activity measurements were normalized to β-

galactosidase values. Measurements were averaged from a series of three (GT1-7 cells) and four (CHO cells) double independent transfection experiments.

The exact constructs used in the transfection experiments were also used for the EMSAs. The 230 nt probes were radiolabeled by PCR with [α^{32}P] dTTP (Amersham-Pharmacia, Piscataway, NJ) and cleaned by passage through Centri-spin-20 columns (Princeton Separations, Adelphia, NJ). Nuclear extracts were obtained from GT1-7 and CHO cells, as previously described (20). Binding reactions were carried out in buffer {20 mM HEPES-KOH pH 7.9, 25% v/v glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 300 μg/ml BSA}, 870,000 cpm of radiolabeled probes, 1.5 μg of poly[d(I-C)], and 5 μg of nuclear extract in a total volume of 25 μl. 50-fold excess unlabeled competitor probes were used for competition on ice, for 15 min, prior to addition of the radiolabeled probes. Reactions were incubated for 15 min at room temperature and stopped by addition of 5 μl of sample buffer (10 mM dithiothreitol, 5% v/v glycerol, and 0.01% w/v bromophenol blue). Samples were resolved on 5% nondenaturing acrylamide-bissacrylamide gels in 1x TBE buffer (run at 120 V for one hour at 4ºC). Gels were dried, and DNA-protein complexes were visualized by autoradiography. The fold-difference between the (–148/+82)*C/C* and the (–148/+82)*T/T* gel shift assays was measured with the NIH IMAGE program and normalized by the free probe at the bottom of each lane.

Initial screening for polymorphisms was carried out using a representative cohort of 25 individuals consisting of diabetic, obese, and lean Caucasians and African Americans. The –38C>T polymorphism was scored by PCR amplification and restriction digestion of the amplicons with the *Aci I* enzyme. PCR cycling conditions were as follows: a denaturation step for 5 minutes at 95 ºC followed by 35 cycles, each cycle consisting of three steps; 95 ºC for 45 seconds (sec), 57 ºC for 45 sec, 72 ºC for 45 sec, and an additional extension step at 72 ºC for 5 minutes. All PCR products and restriction digests were electrophoresed on 3% agarose gels supplemented with ethidium bromide and visualized under UV light. The polymorphism was only found in Africans and Africans of the Diaspora. Hence, three genetically similar populations from three different geographic locations were examined: the Sierra Leoneans from West Africa, the Jamaicans from the Caribbean Sea, and the Gullah-speaking African Americans from the islands and the coasts of South Carolina and Georgia. Populations were sampled on location except for the Gullahs that were sampled at local health centers in South Carolina. BMI was calculated by the weight in kilograms divided by the height in meters squared. Diabetes status was determined by fasting glucose levels ($\geq$ 126mg/dL fasting plasma glucose = diabetes). Institutional Review Board (IRB) approvals for these studies were issued by the Medical University of South Carolina and the corresponding organizations in Jamaica and Sierra Leone. Participants signed Consent Forms.

The SAS program was used in all statistical analyses. Analysis of variance was performed to examine for differences in the mean BMIs between the three genotypes,

each population. The Student's T-test was used to examine for differences in the mean BMIs between the diabetic and the non-diabetic Sierra Leoneans. A Bonferroni correction was made for multiple pairwise testing, which lowered the p-value for significance to 0.025. The contingency χ^2-test was performed to examine for genotype frequencies between genotypes in cases and controls.

Results-Discussion

We have identified upstream nucleotide sequences that contain a putative CCAAT-box and a 21 nt sequence that is 100% identical to the sequence in the promoter of the neuron-derived orphan receptor-1, NOR-1, that is highly expressed in the pituitary, the adrenal, and the rat fetal forebrain. This is similar to *hAGRP* that expresses differentially in the hypothalamus and the adrenal. Algorithmic analysis of the promoter revealed a putative binding site for the Signal Transducers and Activators of Transcription (STATs), at position –122/–114. STAT transactivators have binding sites for the long isoform of the leptin receptor and are potential mediators of leptin's anti-obesity effects. It is of interest that POMC, the precursor of α-MSH that is antagonized by AGRP at the melanocortin receptors, also has recognition sites for the STAT transactivators in its minimal promoter.

A polymorphism was identified at position –38 (–38C>T) and promoter activity of the polymorphic region was evaluated by transfection of the Chinese Hamster Ovary (CHO) and the mouse hypothalamus neuronal GT1-7 cell lines by two constructs that differed only by the polymorphic alleles. The (–148/+82)*C/C* construct had significantly higher luciferase activity than the (–148/+82)*T/T* construct, in both cell lines. Electrophoretic mobility shift assays showed that the binding affinity of transcription factors to the (–148/+82)*C/C* construct was 4-fold and 13-fold higher in the CHO and the GT1-7 cell lines (respectively) than to the (–148/+82)*T/T* construct. Furthermore, algorithmic analysis of the promoter region revealed that the recognition site for the helix-loop-helix (E-box) transcription factors was abrogated at the site of the polymorphism when substituting the cytosine at position –38 by the thymidine. All lines of evidence indicate that the *T/T* genotype at position –38 reduces promoter functions and alters the characteristics of a region that could affect the expression levels of the gene. Cross talk between hormonal/dietary cofactors and cis-acting response elements spanning position –38 and the STAT putative binding site at position –122/–114 may exemplify a regulatory mechanism for *hAGRP* expression levels in the hypothalamus.

To evaluate the possible involvement of the polymorphic site in obesity and type 2 diabetes, the frequency of the –38C>T polymorphism was evaluated in a wide range of populations. There was a significantly higher frequency of the *C/C* genotype in the Sierra Leoneans with high BMI or type 2 diabetes. The higher frequency of the *C/C* genotype in overweight and diabetic Sierra Leoneans parallels the higher promoter activity and affinity for transcription factors of this genotype and suggests that this genotype may augment *hAGRP* expression and increase appetite, possibly, leading to

body-weight gain and type 2 diabetes. Leptin downregulates expression of the murine *AGRP*. However, typical high fat Western diets (30) are commonly associated with leptin-resistance. It is possible, therefore, that *hAGRP* may not respond to leptin's downregulatory effects in the high fat Western diets, irrespective of the promoter genotype. This may not be the case though in the low fat African diets where the *T/T* genotype could provide the means for reduced *hAGRP* expression and possibly appetite (hunger)-resistance during obligatory fasting conditions such as famine. The –38C>T polymorphism in the promoter of *hAGRP* could play a significant role in the regulation of appetite and energy homeostasis in humans but its impact may depend on dietary habits.

Experiments are now underway to determine the transcription factor(s) that bind to the polymorphic site, and algorithmic predictions indicate that those may be of the E12, E47 helix-loop-helix type of transcription factors. In addition, we intend to perform supershift assays to examine whether the STAT transactivators bind to the (–148/+82) probe and if they interact with the E-box transcription factors. In addition, we intend to identify the hormonal regulators (leptin, insulin, ghrelin, etc.) that might affect expression of *hAGRP* in the brain as well as in the periphery.

Experiment 2. A Role for the Human Resistin Gene in Insulin Resistance - Steve Smith, Fulu Bai, George Argyropoulos

Introduction

Recently, a novel 12.5 kD cysteine-rich protein, termed Resistin, was shown to be secreted by adipocytes. *Resistin* gene expression was induced during the differentiation phase of the NIH 3T3-L1 cells. Resistin was downregulated in mature adipocytes exposed to thiazolidinediones (TZD), which are high-affinity ligands for the nuclear receptor peroxisome proliferator activated receptor-γ (PPARγ). Administration of Resistin to wild-type mice impaired glucose tolerance and insulin action, while the levels of the protein were increased in genetic or diet-induced forms of obesity. In contrast to these data, another group reported that *Resistin* expression was decreased in obese mice, increased in ob/ob mice and Zucker diabetic (ZDF) rats in response to PPARγ agonists.

The present study was designed to examine for the presence of genetic variants (polymorphisms) in the promoter of *Resistin*. As in the case of *hAGRP*, it is hypothesized that polymorphisms in the promoter might affect the binding of transcription factors that would otherwise up- or down-regulate expression of the gene. This study is conducted in collaboration with Dr. Steve Smith (at the PBRC). Dr. Smith had already measured the expression levels of *Resistin* by real-time RT-PCR in adipose biopsies from study participants.

Materials and Methods

Human Genome database queries lead to the identification of a polymorphism in the minimal promoter of Resistin. The polymorphism results in a change of a guanine (G) to a cytosine (C). The exact position of the polymorphism has not been determined yet since neither primer extension nor 5' RACE experiments have been performed to confirm the transcription start site(s) in RNA from adipocytes. DNA was isolated from buffy coats from the 58 individuals that had provided the fat biopsies. DNA isolation was performed using a standardized commercial kit (Gentra Systems, Minneapolis, MN). Genotyping of the polymorphism in the 58 samples was performed by PCR amplification of the DNA and restriction digestion of amplicons with a restriction endonuclease.

Constructs to examine promoter activity were designed and directionally cloned (with *Sac I* and *Xho I* restriction recognition sites incorporated into the primers) into the pGL3 luciferase basic vector (Promega, Madison, WI). The two polymorphic constructs were generated by amplification of genomic DNA from a homozygous (*C/G*) individual and the cloning of each strand. No cell transfections have been performed yet but we intend to use two cell lines: the adipocyte NIH 3T3-L1 and the COS-7 monkey kidney cell lines. However, nuclear extracts have been prepared from the two cell lines already and EMSAs have been carried out with the two polymorphic constructs (EMSAs were performed as described above for *hAGRP*).

Results-Discussion

Fifty-eight individuals have been genotyped for the promoter polymorphism and we have identified both *C/G* heterozygotes and *G/G* homozygotes. Interestingly, no *C/C* homozygotes have been detected yet. Preliminary EMSAs have been performed and indicate that there are significant differences in the binding affinities between the two genotypes. Algorithmic analyses have lead to the identification of multiple putative recognition sites for transcription factors throughout the promoter region. We intend to narrow down the list of the candidate transcription factors and single out the ones that are likely to be regulating the expression of *Resistin* in adipocytes.

Supershift assays are being planned out using antibodies for the transcription factors that could interact with the polymorphic site. Statistical analyses are underway to examine whether the Resistin expression levels measured by real-time RT-PCR are associated with the promoter polymorphism.

III. Key Research Accomplishments

- During this transition year we have been successful in recruiting the necessary faculty positions to undertake our new research themes including the metabolic understanding of energy balance, the influence of dietary composition on training

and performance, the prevention of obesity and the development of a functional genomics laboratory.

- The protocol for Project A was submitted to the HSRRB. It has been approved by the PBRC IRB

- The protocols for Projects B and C are being developed.

- For Project D we have accomplished the following

  -Identification and characterization of the promoter of *hAGRP*

  -Identification and functional characterization of a polymorphism in the promoter of *hAGRP*

  -Association of the polymorphism in the promoter of *hAGRP* with obesity and type 2 diabetes in Africans

  -Identification of a common polymorphism in the promoter of the *Resistin* gene

  -Functional characterization of the polymorphism in the promoter of the *Resistin* gene

IV. Reportable Outcomes

Publications funded by Army Grant

1. Smith SR, Lovejoy JC, Greenway F, de Jonge L, et al. Contributions of total body fat, abdominal subcutaneous adipose tissue compartments, and visceral adipose tissue to the metabolic complications of obesity. *Metabolism* 50:425-435, 2001.

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5. Brown AM, Mayfield DK, Volaufova J, Argyropoulos G. The gene structure and minimal promoter of the human agouti related protein (submitted), 2001.

Presentations

1. Argyropoulos G. The Genetics of the Human UCP3 Gene". Experimental Biology Meetings, Special Section: Genomics and Molecular Basis of Exercise & Environmental Physiology. Invited oral presentation, Orlando, FL, 2001.

2. Mayfield DK, Brown AM, Page GP, Argyropoulos G. A role for the agouti related protein in human obesity. American Diabetes Association. Poster presentation, 2001. Philadelphia, PA.

Recent Publications Influential in Planning for the Task

1. **Larson-Meyer DE**, Newcomer BR, Hunter GR. Influence of diet and endurance running on intramuscular lipids in women: a ^{1}H NMR study (in review), 2001.

2. **Larson-Meyer DE**, Newcomer BR, Hunter GR, Weinsier RL, Bamman, MM. Relation between in-vivo and in-vitro measurements of skeletal muscle oxidative metabolism (in review), 2001.

3. Newcomer BR, **Larson-Meyer DE**, Den Hollander J. Intramuscular lipid measurements at 4.1 T by spectroscopic imaging (in review), 2001.

4. Newcomer BR, **Larson-Meyer DE**, Hunter GR, Weinsier RL. Skeletal muscle metabolism in overweight and post-overweight women: an isometric exercise study using ^{31}P magnetic resonance spectroscopy. *Int J Obes* (in press), 2001.

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V. Conclusions

This transition year has seen a switch in paradigm. The typical acute dietary intervention to enhance performance has been modified to more basic research efforts

trying to identify new metabolic pathways involved in the variability in response to diet and physical activity in people. We are excited at the prospective of initiating new innovative studies using state-of-the-art methods including respiratory chambers, doubly labeled water, stable isotope turnovers, and genomics in these upcoming studies. Research conclusions must await the performance of those physiologic studies of Projects A, B, and C.

Project D has produced the following research conclusions.

Our work with the AGRP and Resistin genes is expected to elucidate the genetic pathways that regulate food intake and the utilization of energy stores in humans. This research provides insight into the physiologic mechanisms by which the body regulates weight and into an understanding of how some individuals may be genetically predisposed to weight gain or to resist weight gain. Advancing scientific knowledge in this area is critical to the military interest, since overweight impacts performance negatively. Furthermore, overweight is a prevalent military condition, since failure to meet weight standards currently occurs in ~5% of career military personnel each year.

We draw the following conclusions from our research with the promoters of the human *AGRP* and *Resistin* genes:

- The region –628/+372 contains several promoter consensus sequences and has regions with significant promoter activities.

- Identification of the STAT response element in the promoter of *hAGRP* provides, for the first time, evidence of an end point for leptin's action in the hypothalamus. It appears that leptin follows a pathway that goes from its receptor to the STAT transactivators which then bind to the *hAGRP* promoter to exert leptin's downregulatory action on *hAGRP* expression.

- A common polymorphism –38C>T was identified but was present only in Africans and Africans of the Diaspora.

- The *C/C* genotype of the –38C>T polymorphism had significantly higher promoter activity than the *T/T* genotype.

- The *C/C* genotype had significantly higher affinity for transcription factors than the *T/T* genotype did, when part of a long promoter construct.

- The *C/C* genotype was present at significantly higher frequencies in obese and diabetic Africans suggesting that it might be involved in the genesis of obesity and type 2 diabetes.

- The *C/C* genotype might result in elevated expression levels of *hAGRP* that might

lead to increased appetite in humans.

- The *T/T* genotype may provide a means for satiety-resistance during diminished availability of food.

- The possible effect of the *T/T* genotype on the reduction of appetite might be compromised in high-fat Western diets due to the associated leptin resistance (i.e. the downregulatory effect of leptin on *hAGRP* might be compromised or eliminated in high fat diets).

- A polymorphism was identified in the promoter of the newly discovered hormone Resistin, and it might have a significant impact on the expression levels of the hormone in adipose tissue.

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TASK 5 – NUTRIENT DATABASE INTEGRATION LABORATORY

I. Introduction

Assessing dietary intake is essential in determining the soldier's nutritional needs and how those needs interface with other aspects of military performance. PBRC currently participates in field studies planned and conducted by the Military Nutrition Division of USARIEM by providing assistance with and analysis of dietary intakes collected during military field studies. That participation includes the following:

- Support for USARIEM field studies requiring data collection and data entry needs
- Support for PBRC in-house Military Nutrition Tasks
- Continued programming efforts directed toward meeting computer needs of both

USARIEM and PBRC Military Nutrition Tasks

Previous participation has included the following studies which were conducted as part of the old Menu Modification task (1) and prior to the development of the current Task 5—Nutrient Database Laboratory:

- Fort Jackson, SC, March 1993. Nutritional Intake of Female Soldiers During the U.S. Army Basic Combat Training
- 29 Palms, CA, October 1994. Nutritional Intakes of Marines in Hot Weather
- Fort Polk, LA, March 1995. The Menu Modification Consumption Study
- Fort Sam Houston, TX, July-August 1995. Iron Status of Female Soldiers in Advanced Training

Participation in field studies since the initiation of the Nutrient Database Laboratory Task have included the following studies:

- Hunter Army Airfield & Ft. Stewart, GA, July-Aug 1996. Nutritional Assessment of U.S. Army Rangers in Garrison and During a Field Training Exercise in a Hot, Humid Environment
- Fort Bliss, El Paso, TX, Sept 1996-March 1997. Nutritional Assessment and Dietary Education of U.S. Army Sergeants Major Academy Students in Garrison
- Camp Mackall, NC, April - May 1997. Assessment of Nutritional Status and Energy Expenditures and Determination of Gender Differences in Dietary Intakes of Combat Service Support Personnel Subsisting on Meal-Focused Versions of the Meal, Ready to Eat
- Ft. Bragg, NC, Sept 1997. Impact of Creatine Intake on Physical Performance
- Ft. Lewis Washington, Feb 1998. The Effects of Carbohydrate Supplementation on the Performance of Combat Relevant Activities.
- Bahamas, April-May 1998. Effects of Tray Ration Consumption During a 63-Day Marine Field Exercise
- San Diego, CA, Feb–March 2000. Assessment of Energy Expenditure and Nutritional Status of Navy Women Aboard Ship

In these past studies we have advised the principal investigators in the planning of dietary collection protocols and subsequent analysis of collected dietary intake data.

We continue to work with USARIEM nutrition staff, data programmers, and other key personnel in the development of a shared database system for the collection and processing of dietary information.

II. Body

During the 2000-2001 year, we continued the refinement of a software program to organize the data collected in garrison and field efficiently and quickly (System dubbed MiDAS, Military Diet Assessment System). This replaces the old USARIEM "CAN" system. We also supported USARIEM-sponsored field studies, sending PBRC research associates and senior level dietetics majors as data collectors, as well as programming personnel, for field studies.

PBRC participated in the Ft. Carson study entitled "A Comparative Study Between Special Forces Soldiers and Other Trained Army Units: Assessment of Dietary Intake and Energy Balance in Garrison and Evaluation of Nutritional and Other Risk Factors for Injuries and Illnesses. This study was conducted in July, 2000. The PBRC individuals supporting this study included the following individuals from the Nutritional Epidemiology: Claire Fontenot, worked as recipe specialist; Calynn Davis, worked as recipe specialist; Jarrett Keller, worked as recipe specialist; Eric LeBlanc, data entry; Ray Allen, data entry design, set-up, and entry; Catherine Champagne, data collection advisor.

Eric LeBlanc, data entry, traveled to Fort Drum, NY to support a study conducted by Natick Labs. The study took place from October 29 through November 8, 2000. Dr. Ray Allen and Eric LeBlanc developed the data entry system for this project, which was structured after the Bahamas Study conducted in 1998.

Data from the studies carried out in February and July of 2000 were processed and transmitted to USARIEM. For the February 2000 Essex study, 4108 lines of data were entered on 31 subjects; for the July 2000 Fort Carson study 7522 lines of data were entered on 50 subjects; and for the October Fort Drum study, 13880 lines of data were entered on 137 subjects of a total subject population of 225.

Catherine Champagne and Ray Allen visited USARIEM on April 9 and 10 to discuss logistics for the Parris Island Marine Study which is planned for May-August, 2001. During the visit, Ray Allen also provided assistance in setting up a new computer server at USARIEM for nutritional applications.

PBRC approved funding to support six staff to participate for varying lengths of time for each of three phases (May-June, June-July, August) of the Parris Island, South Carolina study "Assessment of Weight Status and Attrition of Female Marine Recruits during Recruit Training). Dawn Turner and Calynn Davis attended a training on the visual estimation method of dietary assessment held at USARIEM May 21-23, 2001. The

Parris Island study began at the end of May, 2001 and will wrap up at the end of August, 2001. Those participating in this study included, as of this report, Catherine Champagne (oversight of data collection and entry regarding nutritional analysis), Ray Allen (developer of data entry software and system set-up for data entry), Dawn Turner (data collection-visual estimation), Calynn Davis (data collection-recipe specialist), Eric LeBlanc (data entry), and Gina Frazier (data entry).

A summer student, Cameron Lefevre, was hired to assist Ray Allen in website designs that will help to enable the potential communication between PBRC and USARIEM personnel in accessing nutrient information data necessary for field studies.

III. Key Research Accomplishments

- Continued refinement of MiDAS
- Support of data acquisition in field studies developed by USARIEM
    - Ft. Carson, July 2000
    - Ft. Drum, October 2000
    - Parris Island, May 2001

IV. Reportable Outcomes

Publications

1. Champagne CM, Hunt AE, Cline AD, Patrick K, Ryan DH. Incorporating new recipes into the Armed Forces Recipe File: determination of acceptability. *Military Medicine* 166:2:184-190, 2001.

V. Conclusions

The Nutrient Database Integration Laboratory provides essential services for military operations. This Task oversees the operation of MiDAS, the database containing nutrient information for all operational rations. The Task provides critical support to studies which seek to improve soldier nutrition in a variety of field settings.

Future plans for Task 5 include the following efforts:

- MiDAS programming will be continued using state of the art data programming
- We will continue to provide personnel for data collection efforts for USARIEM field studies
- We will prepare for support of studies outlined in our new grant application

- We plan to integrate all Armed Forces Recipes, special formulations (MREs) and other food formulations merged into one centralized database system at PBRC to be maintained and updated for USARIEM's use

VI. References

1. Champagne CM, Hunt AE, Cline AD, Patrick K, Ryan DH. Incorporating new recipes into the Armed Forces Recipe File: determination of acceptability. *Military Medicine* 166:2:184-190, 2001.

TASK 6 – ENHANCING MILITARY DIETS

I. Introduction

This task is designed to evaluate methods to enhance the diets of soldiers in Basic Combat Training (BCT), at Ft. Jackson, SC. Two primary research projects have been planned. One of these research projects has been completed and the other is scheduled to begin, after approval from the Army Institutional Review Board (IRB). Four ancillary studies have also been completed. The primary purpose of this series of studies is to test methods for increasing the consumption of foods rich in calcium, folate, and antioxidant nutrients, i.e., fruits, vegetables, grains, and milk products. The research projects are designed to modify the dining facilities of BCT and/or the food selections of BCT soldiers. One question that was addressed in the first series of studies is how to measure food selections and food intake of soldiers in BCT. The schedule of BCT has a very fast pace; measurement of eating behavior in this environment required the development of innovative techniques to measure food selections and food intake. The study that is scheduled to begin will test the efficacy of two strategies for modifying the food selections of soldiers using a controlled research design. The findings of this study could have significant implications for the design of future dining facilities and for the education of soldiers about healthy nutrition.

II. Body

Study 1. This project was completed during the reporting period 1999-2000. During the current reporting period, the results of this study were prepared for publication and submitted to *Military Medicine*. We recently received the editorial review for this paper and we were invited to revise the manuscript and resubmit. We are optimistic that a revision will lead to a decision to publish the paper. A copy of the paper that was submitted is shown in the Appendix section for Task 6, labeled Appendix A. We plan to submit a revised paper within the next two months.

When planning this study, we observed that there was not a validated measure of stress during BCT. We designed an ancillary study to develop a reliable and valid

measure of stress experienced during BCT. The data of this study have recently been analyzed. We developed a self-report questionnaire that yields a total stress score and has six subscales that measure specific stressful aspects of the BCT experience. A manuscript that presents the research data and describes the new measure can be found in Appendix B. This manuscript will be submitted for publication in the next quarter.

One of the challenges of this series of studies was measurement of food selections and food intake of soldiers in BCT. To accomplish this task, we developed a method called the digital photography method. This method was described in the previous reporting period and is described in detail in Appendix A. Briefly, this approach involves taking digital photographs of food before and after eating and later, in the lab, analyzing these photographs using expert judges and nutrient databases. An alternative method, that has been used in previous military nutrition research involves direct observation of foods by human observers in the dining facility. In Study 1, we found the agreement among judges about food types and amounts to be very high. However, this study did not address the validity of the new digital photography method and it did not compare this new approach the standard visual estimation approach. In a series of three ancillary studies conducted in the school cafeteria at Louisiana Tech, the reliability and validity of both methods was tested against foods of known nutrient values. The results of the first study have been analyzed and are summarized in Appendix C. This is an abstract that was submitted to the North American Association for the Study of Obesity (NAASO). This study found that both methods were valid and that with very large portion sizes, the visual estimation method is more accurate than the digital photography method. The data from the second study will be analyzed within the next two months and the data for a third study will be collected in August-September, 2001. These studies will investigate the validity of the two methods in environments similar to the dining facilities of BCT.

Study2. The second study has been planned. The research protocol can be found in Appendix D. This study compares two strategies for modifying the food selections of soldiers in BCT. Increased consumption of fruits, vegetables, grains, and milk products was the target of the intervention study. The overall program that will be tested is called the Power Up program. One approach for modifying food selections is to provide specific nutrition information in the dining facility. We called this approach "Nutrition Information." Another approach is to educate the soldiers in BCT and the Drill Sergeants about healthy nutrition and to reinforce the selection of fruits, vegetables, grains, and milk products. This approach is called "Nutrition Education." In one dining facility, only the Nutrition Information component will be introduced. In a second dining facility, both the Nutrition Information and the Nutrition Education components will be introduced. The details of this research project are described in Appendix D.

This protocol was approved by the PBRC IRB in October, 2000. It was submitted

to the Army IRB in November, 2000. We are hopeful that it will be approved soon so that the study can be conducted in October-December, 2001.

III. Key Research Accomplishments

- Finding that soldiers in BCT do not eat adequate amounts of fruit
- Finding that overweight soldiers in BCT tend to lose body weight, but lean soldiers tend to gain body weight over the eight weeks of BCT
- Finding that the use of modern digital photography in combination with computer technology and nutrient databases can be used to efficiently and reliably measure food selections and food intake of soldiers
- The perceived stress of BCT can be objectively measured and that the perceived stress of BCT is lessened over the course of BCT
- There is considerable time pressure for soldiers in BCT to complete meals, but soldiers adjust to these time constraints and consume adequate amounts of food

IV. Reportable Outcomes

- Development of the Digital Photography method of measuring food selections and food intake
- Submission of one manuscript to Military Medicine that is undergoing revision
- Presentation of one paper at the 2000 meeting of NAASO
- Submission of one paper to the 2001 meeting of NAASO
- Presentation of the findings of Study 1 in a video teleconference to military personnel at TRADOC, Ft. Jackson, USARIEM, Ft. Bragg and the Office of the Surgeon General
- Presentation of the findings at TRADOC headquarters at Ft. Monroe
- Presentation of the findings at Ft. Jackson
- Presentation of the findings and implications of the findings to the Committee on Military Nutrition Research and the Institute of Medicine

V. Conclusions

Task 6 provides information that can lead to better nutrition during Basic Combat Training. In this series of studies, the methodology for evaluating food selections and food intake in the BCT environment has been developed and tested. The next step for this task will be to test the efficacy of two approaches for modifying the food selections of soldiers. The implications of potential findings from this intervention study are very significant for the health of soldiers. The BCT experience shapes the attitudes and behaviors of soldiers throughout their military career. One important question is whether it is possible to modify the nutrition habits of these soldiers. Another question is how difficult is the task of modifying the nutrition habits of soldiers. The results of the second study should help answer these important questions. If it is possible to modify the eating habits of soldiers and if the intervention that accomplishes this goal is relatively simple and easily transferred to different environments, then the beginnings of a new behavioral health technology will have been initiated.

VI. References

None.

TASK 7 – STRESS, NUTRITION AND IMMUNE FUNCTION LABORATORY

Following the departure of Task Leader Dave Horohov, DVM, on June 30, 2000, this task has been inactive.

Reportable Outcomes

Although Task 7 has been inactive, the previous work on this project has produced the following article:

1. Horohov DW, Pourciau SS, Mistric L, Chapman A, Ryan DH. Increased dietary fat prevents sleep deprivation-induced immune suppression (in press). *Comp Med*, 2001.

TASK 8 – METABOLIC UNIT PROJECT

This project was inactive during the reporting period.

APPENDICES

APPENDIX - GENERAL

Pennington Biomedical Research Center
LOUISIANA STATE UNIVERSITY

COPY

May 23, 2000

RE: Cooperative Agreement No. DAMD17-97-2-7013

Ms. Sherry Regalado
Department of The Army
USAMRAA
820 Chandler Street
Fort Detrick, Maryland 21702-5014

Dear Ms. Regalado:

In accordance with your letter dated May 6, 2000, enclosed is a revised budget in the amount of $3,476,000 to reflect Year 4's decrease in available funding. A substantial budget decrease was made to Project VII: Stress, Nutrition and Immune Function as you recommended. Other minor budget adjustments were made to other projects.

Should you need any additional information, feel free to contact Mr. Patrick Marquette at (225)763-2515.

Sincerely,

for Patrick Marquette

Mr. Ralph P. Underwood
Associate Executive Director -Administration

Dr. Donna Ryan
Associate Executive Director-
Clinical Science

Enclosure

6400 Perkins Road, Baton Rouge, Louisiana 70808-4124 • Phone: (225) 763-2500, Fax: (225) 763-2525

TASK 1

CLINICAL LABORATORY FOR HUMAN AND FOOD SAMPLES

NONE

TASK II

STABLE ISOTOPE LABORATORY

NONE

TASK III

STRESS, NUTRITION AND MENTAL PERFORMANCE

Changes in Rat Adipocyte and Liver Glucose Metabolism Following Repeated Restraint Stress

JUN ZHOU,[1] MING XIA SHI, TIFFANY D. MITCHELL, GENNADY N. SMAGIN, SONYJA R. THOMAS, DONNA H. RYAN, AND RUTH B.S. HARRIS

Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana 70808

Rats exposed to repeated restraint weigh less than controls even 8 weeks after stress. Stress-induced weight loss is lean tissue, but the post-stress difference in weight between control and restrained rats is lean and fat mass. Whole-body glucose clearance is enhanced 1 day after stress, but adipocyte glucose utilization is inhibited and muscle glucose transport is unchanged. The studies described here demonstrated that glucose transport was increased in both restrained and pair-fed rats, but that glycogen synthesis was increased only in restrained rats, which may account for the improved whole-body glucose clearance. Adipocyte glucose transport was inhibited and adipose plasma membrane β-adrenergic receptor number was increased 1 day post-stress in restrained rats when weight loss was lean tissue, but were not different from control rats 5 days post-stress, when both fat and lean tissue were reduced. Thus, repeated restraint induces reversible changes in adipocyte metabolism that may represent a transition from the catabolic state of stress to a new energetic equilibrium in rats that maintain a reduced body weight for an extended period of time.

[Exp Biol Med Vol. 226(4):312–319, 2001]

Key words: glucose transport; glycogen; β-adrenergic receptor

We have previously demonstrated that rats exposed to restraint stress maintain a reduced body weight for extended periods of time during the post-stress period. The initial weight loss is associated with hypophagia on the days of restraint and there is no evidence of compensatory over-eating during the post-stress period to make up for the stress-induced energy deficit (1); therefore, although the rats gain weight after stress, they do not catch up to the weight of non-stressed controls (1). Repeated exposure of rats to restraint stress causes similar, but greater, changes in body weight and food intake (2, 3). Elucidating the mechanism of sustained post-stress weight loss and the temporary inhibition of food intake will assist our understanding of how stress disrupts homeostasis, and may identify mechanisms that are essential for the regulation of body weight in rats.

The weight loss in rats exposed to repeated restraint stress (3 hours restraint on each of 3 days) is exclusively lean body mass 1 day after the last restraint stress, but 5 days after the end of restraint the difference in weight between stressed and control animals is composed of both lean and fat tissue (3). This shift in body composition could only be achieved by tissue-specific changes in nutrient utilization in the days immediately following the end of stress. An oral glucose tolerance test performed 1 day after the end of restraint indicated that whole-body glucose clearance was increased. This change in clearance could not be accounted for by muscle, as glucose transport was not changed in this tissue, or by adipose tissue, because adipocyte glucose uptake was inhibited in restrained rats compared with controls (2). In the experiments described here we measured hepatocyte glucose utilization to determine whether the liver could account for increased whole-body glucose uptake with a simultaneous inhibition of adipocyte glucose uptake and loss of body fat. The results demonstrate a substantial change in hepatic glucose metabolism, but do not elucidate the primary mechanisms responsible for the metabolic changes in restrained rats.

Additional experiments described here tested the hypothesis that loss of fat during the post-stress period was due to inhibition of adipocyte *de novo* lipid synthesis from glucose. Adipocyte glucose transport and adipose tissue β-adrenergic receptor number were measured both 1 and 5 days after the end of restraint. These time points were chosen based on previous observations of significant shifts in the body composition of restrained rats during this interval of the post-stress period (3).

Materials and Methods

Twelve week old male Sprague-Dawley rats weighing 350 g were obtained from Harlan Sprague-Dawley (Houston, TX) and housed individually in wire mesh cages in a humidity and temperature controlled room (22° ± 2°C, 65%

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to 67% humidity) on a 12:12-hr light:dark cycle with lights on at 07:00 hr. All rats were fed a 40% kcal fat, 16% kcal protein diet for at least 10 days before the experiments started, as we have previously found that a high-fat diet exaggerates the effects of restraint stress on body weight (3). Body weights and food intakes were recorded daily. All food intakes, including those of pair-fed rats, were corrected for spillage. All animal protocols were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

For the repeated restraint stress protocol, rats were placed in Perspex restraining tubes (Plas Labs, Lansing, MI) for 3 hr in the morning for 3 consecutive days. The control and pair-fed rats were moved to the same room as the restrained rats and did not have access to food or water for the period of restraint. Pair-fed rats were fed *ad libitum* before stressed rats were restrained, but were given the voluntary intake of restrained rats from the first day of restraint until the end of the experiment. Experiment 1 was conducted with the rats subdivided into groups and the restraint protocol staggered over 3 days to ensure timely collection and handling of tissue and to facilitate pair-feeding to restrained animals.

Experiment 1. Hepatocyte glucose transport and glucose oxidation and incorporation into fatty acids were measured 1 day after the last restraint stress. In this experiment, glucose transport, glucose oxidation, and glucose incorporation into fatty acids were measured in hepatocytes from control, restrained, and pair-fed rats 1 day after the last repeated restraint stress. Thirty rats were fed the high-fat diet for 11 days and were then divided into three weight-matched groups: control, pair-fed, and repeated restraint stress.

One day after the last restraint stress, all rats were deprived of food for 1 to 1.5 hr, from 08.00 hr, prior to anesthesia (ketamine, 90 mg/kg body weight; Xylazine, 10 mg/kg body weight, intraperitoneally). Hepatocytes from each animal were isolated using the method of Berry (4). An incision was made through the abdominal wall and peritoneal cavity to expose the liver. An inflow cannula was inserted into the portal vein, and a perfusion (10 ml/min) was started with continuously gassed (95% O_2/5% CO_2) perfusion media (Krebs bicarbonate buffer without $CaCl_2$, 5.0 mM glucose, pH 7.45). Blood was washed out by cutting the vena cava and the perfusion was continued for 15 min. Another cannula was inserted into the inferior vena cava below the heart, and the interior vena cava near the kidney was closed with a ligature so that digestion media could be recycled. Thus, the outflow went only through the inferior vena cava below the heart. The liver digestion was started by switching the perfusion media to digestion media (Krebs bicarbonate buffer, 5.0 mM glucose, 2% bovine serum albumin [BSA], and 2 mg/ml collagenase, pH 7.45) and was stopped after 10 min. The liver was removed and cells were dispersed using a glass rod and filtered though 250-μm nylon mesh with wash media (Krebs bicarbonate buffer, 3 mM glucose, 10 mM HEPES, 0.5 mM palmitate, and 2% BSA, pH 7.45). The isolated hepatocytes were washed three times and were resuspended in an appropriate volume of wash media.

For measurement of glucose transport, an aliquot of isolated cells was washed once and suspended in transport media (Krebs bicarbonate buffer, 0.1 mM glucose, 30 mM mannitol, 10 mM HEPES, and 2% BSA, pH 7.45). Cell number was determined using a haemocytometer. Trypan Blue dye exclusion was used to determine the number of viable cells and the percentage cell viability was recorded.

Glucose uptake was measured only in basal conditions because liver glucose transport is insulin independent. One milliliter of each cell suspension was added to 2 ml of transport media containing 0.1 μCi/ml ^{14}C-mannitol and was then incubated for approximately 15 min at 37°C with shaking. Then 0.2 mM 2-deoxy D-glucose (2-DG), 1.0 mCi/mM ^{3}H 2-DG in a 50-μl volume was added and the sample was incubated for exactly 2 min. Triplicate 200-μl aliquots of the sample were transferred to vials, immediately centrifuged (5000–6000*g*) to separate cells from media, and the supernatant was aspirated off to stop the reaction. The incubation was repeated in quadruplicate for each rat. The cell fraction was counted for 2-DG incorporation and corrected for extracellular fluid volume, indicated by ^{14}C-mannitol. Results are expressed as nanomoles of glucose incorporated per 10^6 cells per 2 min.

For measurement of glucose oxidation and incorporation into fatty acids in basal and insulin-stimulated conditions, hepatocytes were suspended in incubation buffer (Krebs bicarbonate buffer, 3 mM glucose, 10 mM HEPES, 0.5 mM palmitate, and 2% BSA, pH 7.5). Triplicate 0.5-ml aliquots of each cell suspension were added to 1.0 ml of media containing 0.3 μCi/ml ^{14}C-glucose with or without 1.0 mU insulin/ml. The flasks were gassed with 95% O_2/5% CO_2, sealed with rubber stoppers carrying center wells, and incubated for exactly 2 hr at 37°C with shaking. The reaction was stopped by adding 0.5 ml of 0.5 M H_2SO_4 to media and CO_2 was collected by addition of 0.2 ml of 1.0 M benzethonium hydroxide to the center well. The center well was transferred to a scintillation vial for determination of $^{14}CO_2$ and the cells were extracted for glucose incorporation into fatty acids, as described previously (5). Results were expressed as nanomoles of glucose incorporated per 10^6 cell per 2 hr.

Experiment 2. Hepatic glycogen synthesis was measured 1 day after the end of restraint. This experiment measured glycogen synthesis in liver slices from control, restrained, and pair-fed rats 1 day after the last repeated restraint stress. Twenty-six rats were fed the high-fat diet for 11 days and were then divided into three weight-matched groups: repeated restraint stress, pair-fed, and control.

One day after the last restraint stress, all rats were food deprived for 1 to 2 hr in the morning prior to decapitation between 09:00 and 11:00 hr. The liver was removed and weighed. Six small slices of liver tissue (50–100 mg) from each rat were obtained using a Stadie Riggs tissue slicer.

The slices were weighed and preincubated for 15 min in 2 ml of Krebs bicarbonate buffer, 10 mM HEPES, 5 mM glucose, 2 mM sodium pyruvate, and 1% BSA, pH 7.45 with or without addition of 2 mU/ml of insulin. The tissue was then incubated for 60 min in 2 ml of fresh media that included 0.5 μCi U-^{14}C-glucose/μmol glucose. Reactions were stopped by transferring tissue to ice-cold saline. After washing twice with cold saline, the tissue was dissolved in 1.0 M NaOH, 66% ethanol. Glycogen (~200 μg) was added to each sample and samples were held at −20°C overnight. The samples were centrifuged at 810*g* for 20 to 30 min and the supernatant was discarded. The pellet was washed three times in cold 66% ethanol. The final pellet was dissolved in water and transferred to a scintillation vial for determination of ^{14}C-glucose. Glycogen synthesis was expressed as nanomoles of glucose per milligram of tissue per hour and as millimoles of glucose per liver per hour.

Experiment 3. β-adrenergic antagonist binding to adipose tissue membranes was measured 1 day after the last restraint stress. Eighteen adult, male Sprague-Dawley rats were divided into three weight-matched groups: repeated restraint, pair-fed, and control. The repeated restraint stress and pair feeding protocols were the same as described above except that rats were restrained on 4 instead of 3 consecutive days.

One day after the last restraint stress, the rats were food deprived for 2 hr at the start of the light period prior to decapitation. Epididymal fat was immediately homogenized in cold buffer (Krebs bicarbonate buffer, pH 7.45, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 1000*g* for 10 min at 4°C. The supernatant was further centrifuged at 13,500*g* for 15 min at 4°C. The final pellet was dissolved in an appropriate volume of homogenization buffer for the determination of protein concentration (BCA protein assay kit: BCA, Pierce, Rockford, IL). The final protein concentration in each sample was adjusted to 200 μg/ml.

For determination of maximum binding activity, duplicate 20-μg aliquots of freshly isolated cell membrane were incubated at 37°C for 30 min in 0.6 ml of homogenization buffer with increasing concentrations of ^{3}H dihydroalprenolol (^{3}H-DHA: 1, 5, 20, 40, 60, 80, 100, and 120 nM.) For nonspecific binding, incubation conditions were the same as maximum binding except that a saturating concentration of 5 mM/ml of propranolol was present in the incubation buffer. The reaction was stopped by adding 0.5 ml of ice-cold homogenizing buffer to the tubes and placing the tubes on ice. Membranes were collected on 0.45-μ m nitrocellulose filters (Millipore, Bedford, MA.) and were washed with 10 ml of cold Krebs buffer. The filter was dissolved in 1 ml of ethylene glycol monoethyl ether (Sigma Chemical Co., St. Louis, MO) and bound ^{3}H-DHA was determined by scintillation counting. Specific binding was calculated by subtracting nonspecific binding from maximum binding.

Experiment 4. Adipocyte glucose transport and adipose tissue β-adrenergic receptor number were measured 1 and 5 days after the last restraint. This experiment investigated post-stress effects on adipocyte glucose transport and adipose tissue β-adrenergic receptor number 1 and 5 days after the last restraint stress. The previous experiments did not reveal any differences in adipocyte glucose transport or β-adrenergic receptor binding in tissue from control and pair-fed rats, therefore, only restrained and control groups were included in this experiment.

Thirty rats were maintained on high-fat diet for 10 days and were then divided into three weight-matched groups: Control, Restraint-1, and Restraint-5. Rats in the Restraint-1 group were killed 1 day after the last day of repeated restraint and rats in the Restraint-5 group were killed 5 days after the last restraint stress. Half of the control rats were killed at a time that was equivalent to 1 day after the last restraint stress and the other half were killed at a time that was equivalent to 5 days after the last restraint stress. The start of the experimental protocol was staggered over 5 days to ensure that animals from all of the different groups were killed on the same day. Rats were food deprived for 1 to 2 hr in the morning before decapitation. Epididymal fat was dissected and weighed, and half of it was used to measure maximal β-adrenergic receptor binding, as described above except that a single concentration of 150 nM ^{3}H-DHA was used. The other half was used to measure glucose transport in isolated adipocytes.

Adipocytes were isolated by the method of Rodbell (6) and were suspended in transport buffer (1× Krebs, 0.1 mM glucose, and 2% BSA). Glucose uptake was measured in basal and insulin-stimulated conditions. Duplicate 1-ml aliquots of each cell suspension were added to 2 ml of media containing 0.1 μCi/ml ^{14}C-mannitol with or without 0.8 mU/ml of insulin, and were incubated for 30 min at 37°C with shaking. Cell number was determined by fixing an equivalent aliquot in osmium tetroxide and counting by Coulter Counter, as described previously (7). Fifty microliters of 0.2 mM 2-DG, 1.0 mCi/mM ^{3}H 2-DG was then added and the sample was incubated for exactly 2 min. Triplicate 200-μl aliquots of the sample were transferred to vials containing 100 μl of phthalic acid dinonyl ester and was immediately centrifuged to separate cells from media. The cell fraction was counted for 2-DG incorporation and was corrected for extracellular fluid volume, represented by ^{14}C-mannitol. Results are expressed as nanomoles of glucose incorporated per 10^{6} cells per 2 min.

Statistical Analysis. Results for hepatocyte glucose uptake, adipocyte glucose transport, and receptor binding in Experiment 4 were analyzed by one or two-way analysis of variance (ANOVA) with *post hoc* Duncan's multiple range test. All other data for liver glucose utilization were analyzed by repeated measures ANOVA with insulin concentration as the repeated measure. The SAS system, version 6.12, was used for computations. The results for receptor binding in Experiment 3 were analyzed by a nonlinear regression model for radioligand binding data (Prism Software, GraphPad Software, San Diego, CA). The B_{max} and

K_d from each group were obtained using the average data from each rat. Data are presented as means ± SEM.

Results

Body Weight and Food Intakes of Rats in the Four Experiments. The restrained rats lost weight on the days that they were stressed, and food intake was inhibited on the 3 days of restraint in all four experiments. In Experiment 4, rats did not return to their pre-stress body weight during the 5-day post-stress period and there was no evidence of overeating to compensate for the stress-induced hypophagia (data not shown). We have previously reported that stressed rats do not reach control body weight even 40 days after the end of repeated restraint (3).

Experiment 1. Hepatocytes from both restrained and pair-fed rats had significantly higher rates of glucose transport than those from control rats ($P < 0.05$), as shown in Figure 1. Hepatocyte glucose oxidation and incorporation into fatty acids were not different among the three groups in either basal or insulin-simulated conditions (Figure 2).

Experiment 2. Restrained rats had significantly higher rates of liver glycogen synthesis in basal conditions compared with control and pair-fed groups, when data were expressed either as nanomoles of glucose incorporated per milligram of tissue per hour or as millimoles of glucose incorporated per liver per hour (see Fig. 3). This significant difference was not apparent in insulin-stimulated conditions because glycogen synthesis in tissue from restrained rats did not increase beyond the levels found in basal conditions.

Experiment 3. Figure 4 clearly shows that at the same ^{3}H-DHA concentration, specific binding of ^{3}H-DHA to adipose plasma membrane was saturated for tissue from control and pair-fed rats, but not from restrained rats. The B_{max} (control, 147 fmol/μg; pair-fed, 167 fmol/μg) and K_d (control, 75 pM; pair-fed, 62 pM) for control and pair-fed rats were similar. Since binding was not saturated for restrained rats, B_{max} and K_d could not be calculated. However, as concentration of ligand needed for saturation is determined by receptor number, it may be assumed that receptor number was increased in restrained rats. Due to large inter-animal variation, we could not compare K_d among the three groups of rats.

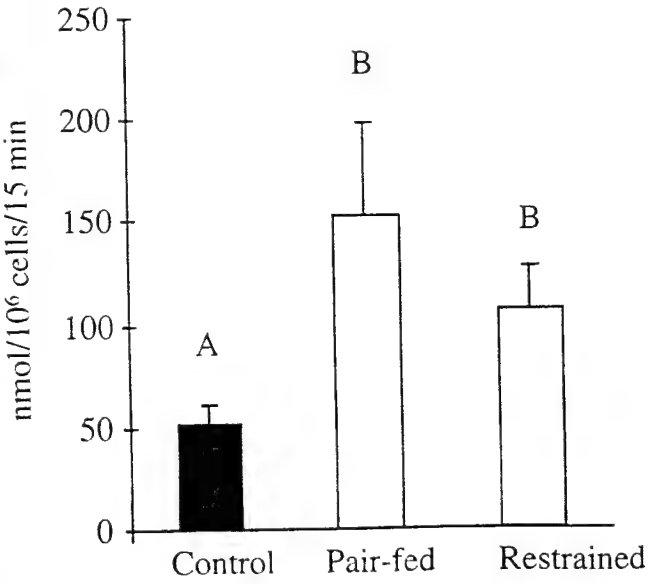

Figure 1. Glucose transport in isolated hepatocytes from control, pair-fed, and restrained rats in Experiment 1. Data are means ± SEM for groups of 10 rats. Values that do not share a common letter are significantly different at P < 0.05. Cells from pair-fed and restrained rats had significantly higher rates of glucose transport than those from control rats.

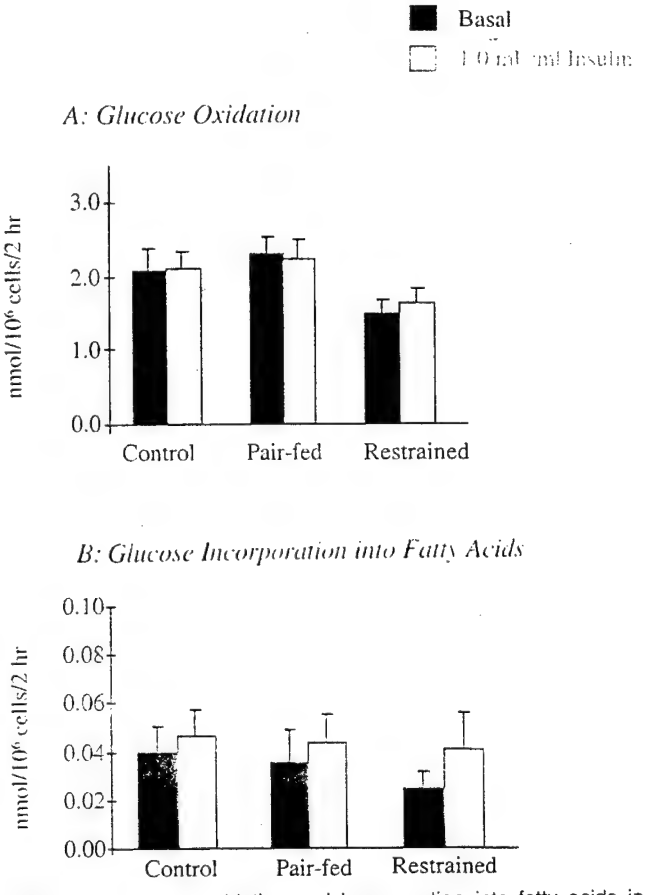

Figure 2. Glucose oxidation and incorporation into fatty acids in hepatocytes from control, pair-fed, and restrained rats in Experiment 1. Data are means ± SEM for groups of 9 rats. There were no statistically significant differences between groups for either measurement.

Experiment 4. Restraint stress ($P < 0.05$) and insulin ($P < 0.05$) had significant effects on adipocyte glucose transport measured 1 and 5 days after restraint, as shown in Figure 5A, but there was no interaction between stress and insulin ($P > 0.1$). Glucose transport in adipocytes from Restraint-1 rats was significantly reduced compared with that in control rats in both basal and insulin-stimulated conditions. Transport in cells from Restraint-5 rats was not different from either the control or Restraint-1 groups. Adipose tissue β-adrenergic receptor binding was significantly higher in tissue from Restraint-1 rats than either control or Restraint-5 rats. ($P < 0.05$), but there was no difference between control and Restraint-5 animals (see Fig. 5B).

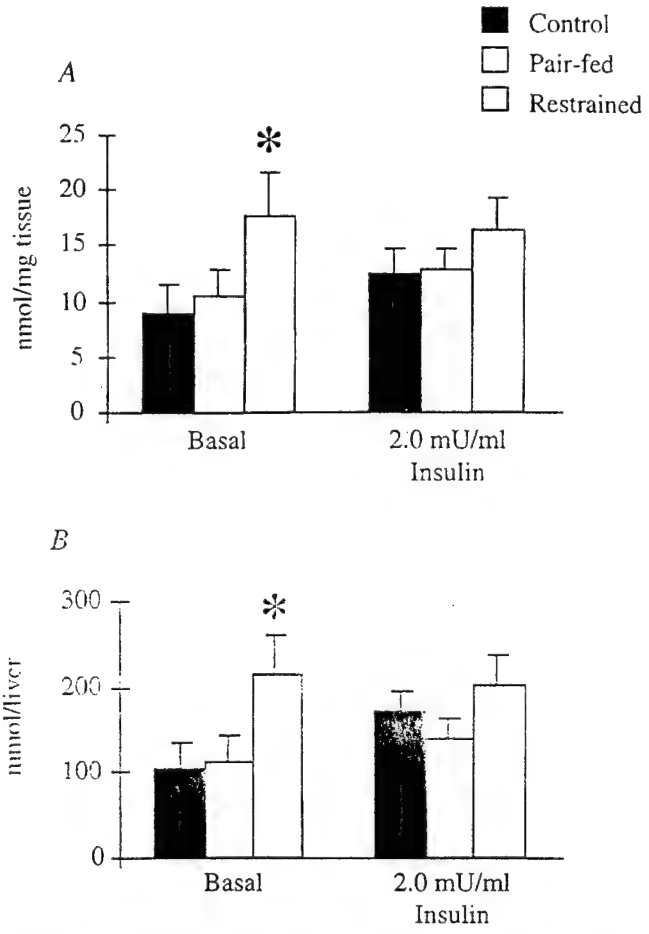

Figure 3. Glycogen synthesis in liver slices from control, pair-fed, and restrained rats in Experiment 2. Data are mean ± SEM for groups of 8 rats. (A) Glycogen synthesis expressed per milligram of tissue. (B) Glycogen synthesis expressed per liver. Glycogen synthesis was significantly higher ($P < 0.05$) in restrained than control or pair-fed rats, as denoted by an asterisk.

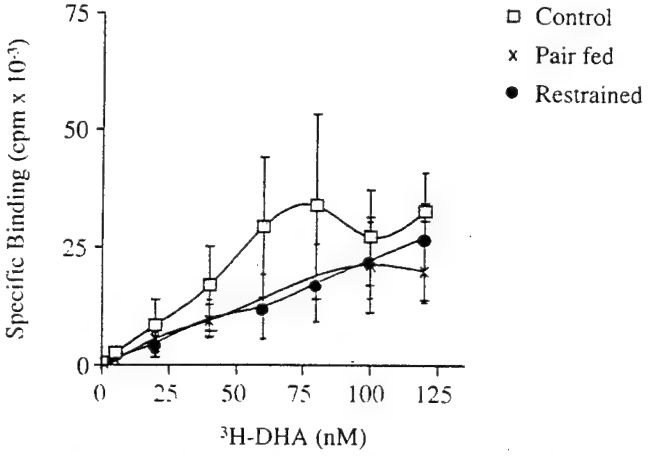

Figure 4. Specific binding of ^{3}H-DHA to adipose plasma membrane of control, pair-fed, and restrained rats from Experiment 3. Data are means ± SEM for 5 or 6 rats per group.

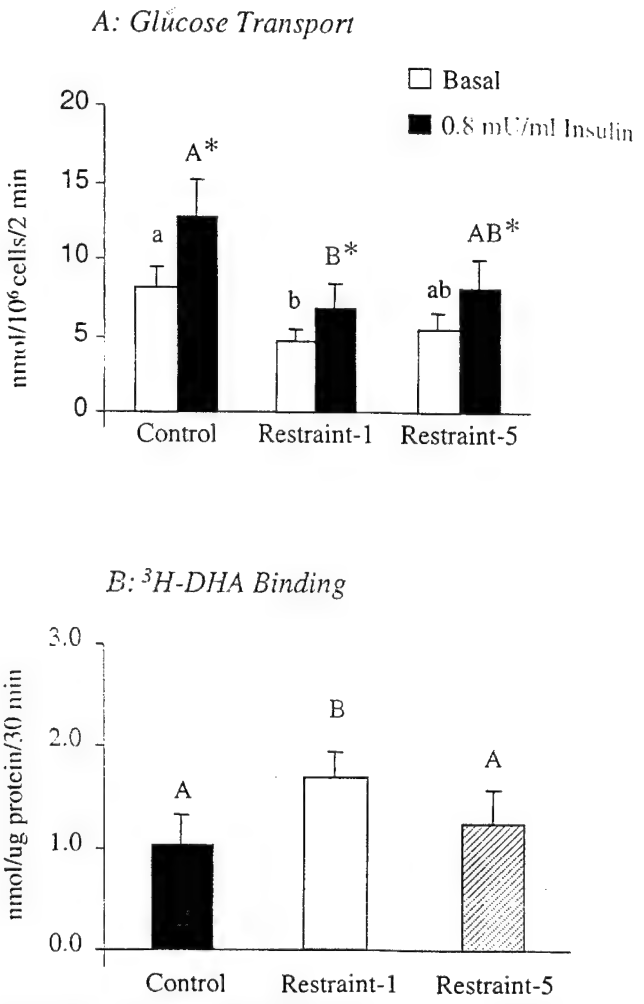

Figure 5. (A) Adipocyte glucose transport in control, Restraint-1, and Restraint-5 groups of rats from Experiment 4. Data are means ± SEM for groups of 10 rats. Insulin significantly increased glucose transport in all three groups of rats ($P < 0.05$), as indicated by an asterisk. Both basal and insulin-stimulated glucose transport were significantly lower in Restraint-1 rats than controls ($P < 0.05$), indicated by lower case superscripts for basal transport and upper case superscripts for insulin-stimulated transport. Restraint-5 rats were not different from either control or Restraint-1 groups. (B) β-Adrenergic receptor binding of ^{3}H-DHA in control, Restraint-1, and Restraint-5 groups of rats from Experiment 4. Data are means ± SEM for groups of 10 rats. β-Adrenergic ligand binding was significantly higher for Restraint-1 than control or Restraint-5 animals ($P < 0.05$). There was no difference between control and Restraint-5 rats.

Discussion

Previous experiments have shown that repeated restraint stress causes a sustained down-regulation of body weight in rats, such that they weigh significantly less than their controls even 40 days after the end of restraint (3). The weight loss during stress is accounted for by catabolism of lean tissue, but within 5 days of the end of stress the composition of weight loss is a combination of lean and fat mass. Whole-body glucose clearance is also significantly improved 1 day after the end of stress despite a substantial inhibition of adipocyte glucose transport and no measurable change in muscle glucose uptake (2). Because the shift in body composition can only be achieved by tissue-specific changes in nutrient utilization, these studies tested the effects of repeated restraint stress on liver and fat metabolism

during the early stages of the post-stress period. All of the measurements were made *in vitro* on isolated cells or tissue slices, and differences between groups represented responses mediated by cellular factors such as levels of protein expression. They did not account for differences between groups that were mediated by acute *in vivo* neural or hormonal input to the cells.

Hepatocyte glucose transport was significantly increased 1 day after the end of restraint in restrained and pair-fed rats compared with controls. This may account for our previous observation of increased whole-body glucose clearance in restrained rats (2). Net hepatic glucose uptake is determined by the balance between glucose uptake and output and it has been shown that the stress of exercise, trauma, or infection stimulates hepatic glucose production (8–10). We did not measure glucose production; therefore, the increased glucose transport into hepatocytes of restrained rats could reflect either an increase in net glucose uptake, if hepatic glucose production was normal, or no net change in uptake if glucose production was increased. We hypothesize that the first situation was true for restrained rats and the second was true for pair-fed rats. There are four alternative metabolic pathways for glucose in the liver: (i) local oxidation to provide energy; (ii) glycogen synthesis to store glucose; (iii) lipid synthesis for energy storage; and (iv) transport out of the liver as an energy substrate for other tissues. Liver glycogen, but not lipid, was increased during the post-stress period in restrained rats compared with pair-fed rats (2) and results from these experiments show that glycogen synthesis was increased (see Fig. 3), indicating an increase in net glucose uptake that is insulin independent. In pair-fed rats, glucose oxidation and glycogen and lipid synthesis were not changed, suggesting that the increased hepatic glucose uptake was associated with increased glucose efflux and no net change in glycogen stores.

Although the liver probably accounts for increased whole-body glucose clearance in both restrained and pair-fed animals, the mechanisms responsible for this increase may be different as the glucose has a different metabolic fate in the two groups. It has been reported that stress hormones such as norepinephrine, epinephrine, and cortisol inhibit insulin release and cause insulin resistance in peripheral tissue (11). If these hormones are chronically elevated, insulin-independent hepatic glucose uptake could increase to keep blood glucose within the normal range. Single time-point measures of brain catecholamines and serum corticosterone immediately following a single 3-hr restraint (1) and serum corticosterone 1 day after repeated restraint (3) were not different between control and restrained animals. The observations imply that changes in hepatic metabolism are not due sustained activation of the Corticotropin-releasing factor or sympathetic systems, but that repeated restraint initiates a cascade of events that effects tissue metabolism even 24 hr after the stress has ended. Single measures of hormone concentration do not exclude the possibility of a change in the circadian pattern of hormone release that alters the balance between anabolic and catabolic hormones and, ultimately, peripheral tissue metabolism. In contrast to restrained rats, pair-fed rats were food-restricted throughout the experimental period and neural or endocrine responses to this on-going stress may have directly promoted hepatic glucose transport.

In 1963, Russek (12) hypothesized that the central "feeding center" could respond to changes in hepatic glucose flux by monitoring the arterio-portal glucose gradient. Niijima (13, 14) subsequently proposed that glucose-sensitive vagal afferent fibers from the liver to the hypothalamus played a role in controlling food intake and others (15–18) have reported that liver glucose metabolism influences feeding behavior. Infusion of glucose into the portal, but not the jugular, vein inhibits feeding and increases liver glycogen content (19) unless the rats are fasted and already have a reduced liver glycogen content (20). Liver glycogen was increased in restrained rats in the post-stress period and the rats did not overeat to compensate for their reduced food intake during stress, consistent with the hypothesis that liver glycogen inhibits feeding. The difference in hepatic glycogen between restrained and pair-fed rats may represent a post-stress response in the restrained rats, but in addition, it was likely that the hungry, pair-fed rats ate their food in large meals early in the day, whereas restrained rats were eating *ad libitum*. This difference in meal patterns would have had an independent effect on liver glycogen content, which in turn may have influenced the appetite of the animals.

Weight loss in rats exposed to repeated restraint is entirely lean body mass during the stress but 5 days post-stress the difference in weight between control and restrained rats is composed of both lean and fat tissue (2), implying a selective inhibition of fat accretion once stress has ended. Experiments 3 and 4 demonstrated that adipose tissue β-adrenergic receptor number was increased in restrained rats the day after stress ended, but was not different from controls 5 days after the end of stress. We measured adipose tissue plasma membrane binding of ^{3}H-DHA, a β-adrenergic receptor antagonist (21, 22) that binds to all three β-adrenergic receptor subtypes, β_1, β_2, and β_3, all of which are present in rat adipose tissue (23, 24). Activation of these receptors would increase lipolysis and decrease glucose transport in adipose tissue (24, 25). Different subtypes of β-adrenergic receptors make variable contributions to these effects depending on species, age, and receptor activity (26–28), but in adipose tissue, β_3-adrenergic receptors are primarily responsible for catecholamine-stimulated lipolysis and acute inhibition of glucose transport (29). In our experiments it would have been preferable to identify binding activity of each receptor subtype individually; however, specific β_3-adrenergic agonists or antagonists were not commercially available.

The increased β-adrenergic receptor number in adipose tissue from restrained rats would enhance the stimulation of lipolysis and inhibition of glucose transport caused by β-ad-

renergic receptor activation (24). Thus, the post-stress inhibition of glucose transport (2) and loss of body fat from restrained rats (3) could be secondary to an up-regulation of β-adrenergic receptors. Under conditions of sustained sympathetic activation, adrenergic receptors are usually down-regulated or desensitized (25, 30). Thus, the increased number of receptors is consistent with previous observations that repeated restraint stress is not associated with chronic activation of the catecholaminergic system (1, 2). The mechanism responsible for an increase in β-adrenergic receptor number in restrained rats needs further investigation.

β-Adrenergic receptor number can be regulated by factors such as corticosterone, TNF-α, and protein kinase C (31–33). Corticosterone has been reported to prevent the down-regulation of β_2-receptors by a β-agonist, so that the combination of corticosterone and β-agonist resulted in no net change in receptor number or gene expression (34). This could also be the case in pair-fed rats, which did not show any differences in β-adrenergic receptor number, although they were under the continuous stress of food restriction and corticosterone was elevated throughout the period of food restriction (2).

In summary, studies described provide additional information on post-stress metabolic changes in rats that have been exposed to repeated restraint. Although these studies do not identify the primary mechanisms responsible for the maintenance of a reduced body weight, they demonstrate a temporary modulation of adipose tissue metabolism during the post-stress period that may account for the changes in body composition that occur within days of the end of restraint. In addition, the results suggest that changes in liver glucose metabolism may contribute to the failure of the rats to overeat when they have an opportunity to compensate for stress-induced negative energy balance. The present studies help to define, on a temporal basis, the contribution of peripheral tissue metabolism to end-point energetic responses during the post-stress period. Future studies will investigate the mechanisms responsible for these chronic responses to repeated acute activation of the Corticotropin-releasing factor system.

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Identification of urocortin mRNA antisense transcripts in rat tissue

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ABSTRACT: Urocortin (UCN) has 45% sequence homology with corticotropin releasing factor (CRF) and binds to CRF receptors. We used reverse-transcriptase-polymerase chain reaction to demonstrate the presence of UCN RNA in various brain regions and in peripheral tissues. Ribonuclease protection assay (RPA) using sense and antisense riboprobes demonstrated the presence of a naturally occurring antisense UCN RNA transcript in a number of tissues. Northern blot indicated that the antisense transcript was the same size as sense UCN. RPA, using probes that covered bases 1 to 560 of 579 bp sequence of rat UCN, indicated that the antisense sequence was complementary to sense UCN but did not contain an open reading frame. Sense and antisense UCN RNA were co-expressed in all tissues that contained levels of either transcript detectable by RPA. Sense RNA expression was greater than antisense in the midbrain, the two transcripts were expressed equally in the hypothalamus and antisense was expressed at higher levels than sense in the liver, heart, and skeletal muscle. Antisense RNA expression was stress responsive, suggesting that it may play a role in regulating transcription or translation of UCN mRNA. © 2000 Elsevier Science Inc.

KEY WORDS: Stress, Ribonuclease protection assay, Muscle.

INTRODUCTION

Urocortin (UCN) is a 40 amino acid neuropeptide that shares 45% sequence identity with corticotropin releasing factor (CRF) and 63% sequence identity with fish urotensin [27]. Intracerebroventricular injection of UCN elevates plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels, suppresses food intake [15], and causes anxiety-type behavior in rats [22]. These responses are typical of those caused by stress or central administration of CRF. UCN binds with high affinity to both CRF type 1 (CRFR-1) and type 2 (CRFR-2) receptors and was initially hypothesized to be an endogenous ligand for CRFR-2 [27], however, a thorough investigation of the co-localization of UCN immunoreactive neurons and CRFR-2 expression indicates that there is not a strong relationship between UCN-containing neurons and cells with CRFR-2 receptors [6].

Despite the similar responses to administration of exogenous CRF and UCN, the two peptides are likely to have unique physiological functions due to their central distribution in relation to CRF receptors [6,16,23]. For example, ACTH is increased by UCN administration in rats, but neutralization of endogenous UCN does not change stress-induced ACTH release which must be dependant upon CRF or neural activation of the pituitary gland [25]. *In situ* hybridization studies demonstrated that UCN mRNA was expressed at high levels in the Edinger-Westphal nucleus, the lateral superior olive, and the supraoptic nucleus, with low levels of expression in the paraventricular nucleus of the hypothalamus, cerebellum, hippocampus, and the anterior pituitary [6,18,29]. Immunohistochemistry demonstrated UCN protein in the Edinger-Westphal nucleus, the lateral superior olive, and the supraoptic nucleus with neural projections extending to areas of the brain and brain stem that control motor function and sensorimotor integration [6]. In contrast, CRF mRNA is expressed at high concentrations in the paraventricular nucleus of the hypothalamus, the central nucleus of the amygdala, the bed nucleus of the straia terminalis, the substantia innominata, and other limbic structures [5].

In peripheral tissue, CRF mRNA and its encoded peptide are expressed in ovary, testis, spleen, thymus, and placenta [3,24]. Studies using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunocytochemical analysis have demonstrated that both UCN mRNA and peptide are produced by human lymphocytes [4], placenta, and fetal membranes [20]. UCN mRNA expression has been detected in stomach, gut, and lymphoid tissue by *in situ* hybridization [6] and UCN immunoreactivity has been reported for the duodenum [27], stomach, colon, and testis [18]. In the current study, we further investigated the tissue distribution of UCN mRNA in rats using RT-PCR and an ribonuclease protection assay (RPA). In the process, we identified the existence of a naturally occurring UCN antisense RNA which is responsive to restraint stress. Natural antisense RNAs are endogenous transcripts that exhibit complementary sequences to their mRNA counterparts [26]. Some antisense RNAs encode proteins [12,26] and others regulate their corresponding sense mRNAs [11,26]. Identification of natural UCN antisense RNA may be important for understanding the transcriptional regulation of the UCN gene.

MATERIALS AND METHODS

Tissue Distribution of UCN PCR Products

Six male Sprague–Dawley rats (Harlan Sprague Dawley Inc, Harlan, IN, USA), weighing 200–300 g, were exposed to 1 h of stress in a restraining tube (Plas Labs, Lansing, MI, USA). Stressed rats were used to optimize the probability of detecting

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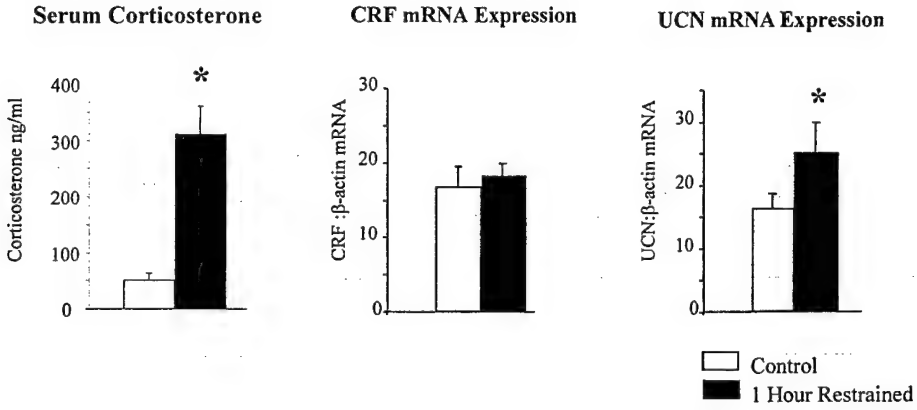

FIG. 1. Serum corticosterone and hypothlamic corticotropin releasing factor (CRF) and urocortin (UCN) mRNA expression in non-stressed (Control) and rats restrained for 1 h. mRNA is expressed as a ratio to β-actin mRNA. An asterisk indicates a significant difference between control and restrained animals ($p < 0.05$), determined by unpaired t-test ($n = 8$).

UCN expression. In a pilot study, rats were restrained for 1, 2, or 3 h and hypothalamic UCN and CRF mRNA were measured by RPA, as described below. One hour of restraint caused a significant increase in hypothalamic UCN mRNA expression (see Fig. 1) which was not increased further by extending the duration of restraint (data not shown). Weninger et al. [28] reported a three-fold increase in UCN mRNA expression in the mid-brain after 3 h of restraint, measured by *in situ* hybridization, but did not measure any earlier time-points. In this experiment, rats were decapitated at the end of restraint and adrenal glands, fat, skeletal muscle, heart, liver, spleen, testis, pituitary gland, hippocampus, hypothalamus, and midbrain were dissected and snap frozen. All animal procedures were approved by the Pennington Center Institutional Animal Care and Use Committee. Total RNA was extracted using Trizol Reagent (Life Technologies, Rockville, MD, USA) and treated with DNase I (Life Technologies) at 1 unit per μg RNA for 15 min at 25°C to remove genomic DNA. RNA was phenol-chloroform extracted and precipitated with ethanol.

UCN gene specific oligonucleotide primers were synthesized based on the published 579-bp rat UCN cDNA sequence (Genebank accession number U33935). Tissue expression of UCN gene was initially assessed using an RT-PCR analysis in which oligodT$(_{15})$ was used for the RT reaction followed by PCR using primers rUCN-P1 and rUCN-P2 (see Table 1) to amplify bases 153 to 403 of rUCN (PCR conditions: 94°C for 5 min; 94°C, 60°C, 72°C each for 1 min for 50 cycles; 72°C for 6 min). An extended number of cycles were performed as an initial PCR with 30 cycles demonstrated the presence of small quantities of amplification product in some tissues that were reliably detected when the number of cycles was increased.

A PCR product of the predicted size (251 bp) was the dominant band for every tissue tested, except for fat. However, there were additional amplification products of varying sizes in a number of the tissues, therefore, Southern hybridization was used to authenticate the PCR products. The PCR products were separated on a 1% agarose gel and transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL, USA). The blot was prehybridized in Rapid-Hyb hybridization buffer (Amersham) for 2 h and then hybridized with a ^{32}P labeled rat UCN cDNA probe at 45°C for 2 h. The probe was made from a 251-bp insert, corresponding to bases 153 to 403 of rUCN, by random priming (Multi-Prime kit; Amersham). The blot was washed in 0.1% standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 65°C for 20 min and exposed to a Phosphorimaging screen for 3 h (Molecular Dynamics, Sunnyvale, CA, USA).

Ribonuclease Protection Assay

The rUCN-P1/P2 PCR product was cloned into pCR2.1 vector by TA cloning (Invitrogen Inc., San Diego, CA, USA). The inserted sequence and orientation was confirmed by sequence analysis (GeneLab, Louisiana State University, Baton Rouge, LA, USA). The RPA was carried out using an *RPA II* kit (Ambion Inc., Austin, TX, USA). Radiolabeled sense and antisense riboprobes were synthesized using T7 RNA polymerase from two different plasmid clones harboring the 251-bp insert in opposing orientations. Unlabeled sense or antisense strand RNA were also transcribed and used as size markers for UCN. Rat CRF antisense riboprobe, was synthesized with SP6 RNA polymerase from a pCRII clone harboring a 356-bp rat CRF insert, and was used to compare levels of expression of CRF and UCN in the same sample. Rat β-actin (Ambion Inc.) antisense riboprobe was used as an internal control to correct for RNA loading. Full-length riboprobes were gel-purified and hybridized with 20 μg total RNA overnight at 45°C. Reaction mixtures were digested for 30 min with RNase A/T1. The reaction was stopped by inactivation buffer

TABLE 1

PRIMERS USED FOR POLYMERASE CHAIN REACTION AND DEVELOPMENT OF RIBOPROBES

Primer	Sequence	Location According to U33935
rUCN-P1	5′-GGCGAATGTGGTCCAGGA	153-170
rUCN-P2	5′-TGATGCGGTTCTGCTGTGC	385-403
rUCN-P3	5′-ATCCAGTCAGAGTGTTCAG	542-560
rUCN-P4	5′-CGCGCACTCCTCTTGCTGTT	220-239
rUCN-P5	5′-CTGAGCCAGCTCCGGTTGTG	492-511
rUCN-P6	5′-GCGGCCGCTCTCCATCTTG	1-19
rUCN-P7	5′-CTGCAGGCTCAGATCCCGC	262-280
rUCN-P8	5′-CCCGCCGTTGTCCATCGAC	303-321

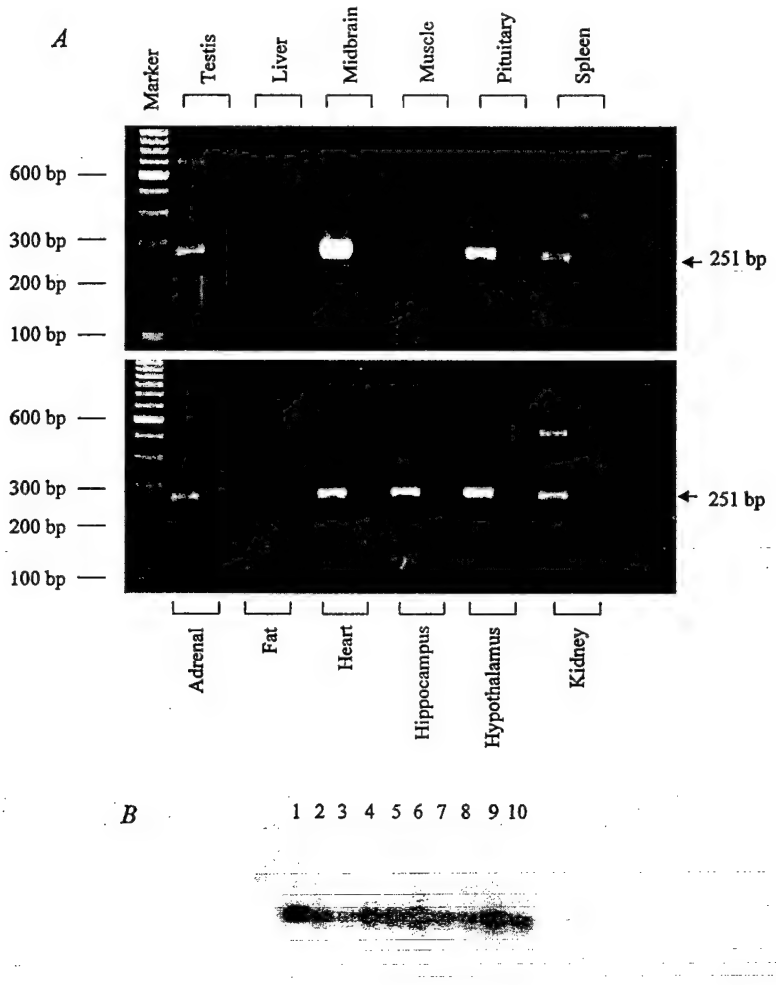

FIG. 2. (A) Reverse transcripate polymerase chain reaction (RT-PCR) of UCN mRNA in rat tissues. Total RNA was reverse transcribed using oligodt [15] and subjected to PCR using primers rUCN-P1/P2. The expected 251-bp product was amplified in all tissues tested. The second of each pair of lanes is a negative control that did not include reverse transcriptase in the RT reaction. (B) Southern blot of PCR products. Lane 1, 251-bp UCN insert; 2, adrenal; 3, cerebellum; 4, kidney; 5, heart; 6, midbrain; 7, muscle; 8, hippocampus; 9, hypothalamus; 10, pituitary.

and protected fragments were precipitated and separated on a denaturing 5% polyacrylamide, 8 M urea gel. The dried gel was exposed to a Phosphorimaging screen for 12–48 h.

The results of the RPA indicated that some tissues expressed antisense UCN RNA. The level of expression of sense and antisense UCN RNA in midbrain, hypothalamus, heart, and skeletal muscle of stressed and non-stressed rats was calculated as a ratio to actin expression, to determine whether both sense and antisense transcripts were responsive to stress. To further confirm and characterize the UCN antisense transcripts, regions of rat UCN corresponding to the 5′, central, and 3′ regions of the 579-bp UCN cDNA sequence were amplified by RT-PCR using primers described in Table 1. Primers rUCN-P6/P7 amplified bases 1 to 280 and primers rUCN-P8/P3 amplified bases 303 to 560. The 5′ and 3′ PCR products were cloned into pCRII by TA cloning (Invitrogen Inc.) and radiolabeled sense riboprobes were synthesized using T7 RNA polymerase from linearized cloned templates of the 280-bp and 258-bp inserts. RPA was performed as described above.

Evaluation of UCN Sense and Antisense Expression by PCR

To distinguish between sense and antisense PCR products, priming of RT reactions with oligonucleotides specific to the sense or antisense strand of UCN was performed. The rUCN-P1 primer was used to synthesize first strand antisense cDNA and the rUCN-P3 primer (see Table 1) was used to synthesize first strand sense cDNA. Total RNA (0.5 μg) from each tissue was reverse transcribed and subjected to PCR using a primer pair (rUCN-P4 and rUCN-P5) that amplified a 292-bp product corresponding to bases 220 to 511 of rUCN. The PCR conditions were: 95°C for 5 min, 30 or 35 cycles of 95°C, 61°C, 72°C each for 1 min and 72°C for 6 min. Reactions with no reverse transcriptase were used as controls.

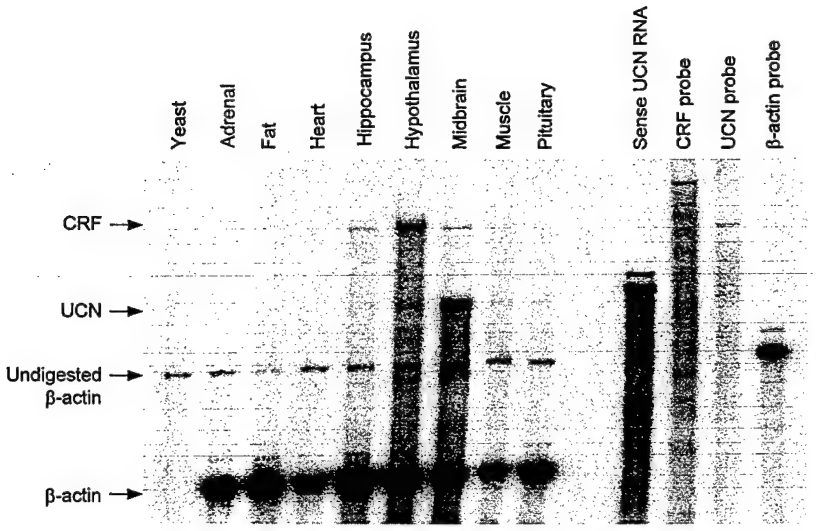

FIG. 3. Tissue distribution of sense urocortin (UCN) and corticotropin releasing factor (CRF) mRNA detected in a ribonuclease protection assay (RPA). ^{32}P- labeled riboprobes for rat UCN and CRF were used, along with β-actin as an internal control. The protected fragments for UCN, CRF, and β-actin are indicated by arrows. Some undigested β-actin probe was present in all samples including the yeast RNA control.

Northern Blot of UCN

Multiple unsuccessful attempts were made to obtain the full sequence of antisense mRNA by rapid amplification of cDNA ends (RACE) (Marathon cDNA Amplification kit: Clontech, Palo Alto, CA, USA; 5′/3′ RACE kit: Boehringer Mannheim, Indianapolis, IN, USA). Finally, the sizes of antisense and sense mRNA were compared by Northern blot. A cDNA probe was made by radiolabeling the 3′ probe, described above. The insert was cut from pCRII with EcoR1 and labeled with ^{32}P dCTP by random priming (Multiprime kit; Amersham Corp.). Total RNA extracted from midbrain (15 ug), heart (20 ug), or skeletal muscle (20 ug) of stressed rats was separated in a 1.2% agarose, 2.2 formaldehyde gel in MOPS buffer, transferred to a nylon membrane (Hybond; Amersham Corp.) and immobilized by exposure to ultraviolet light. The membrane was prehybridized in RapidHyb buffer (Amersham Corp.) at 65°C for 1 h and hybridized with radiolabeled probe (3 ng probe, ~1.5 uCi/ng DNA) for 3 h. The membrane was washed twice with 2 × SSC, 0.1% SDS and exposed to x-ray film overnight. The size of the mRNA hybrids was determined by comparison to an RNA ladder (Millenium marker: Ambion Inc.) run on the same membrane.

RESULTS

Figure 2A shows the results from the initial RT-PCR which produced a 251-bp product in the following tissues: adrenal gland, heart, skeletal muscle, hippocampus, hypothalamus, midbrain, pituitary, liver, kidney, spleen, and testis. No product was detectable in fat. The RT-PCR was not quantitative and the large number of cycles resulted in the appearance of additional amplification products in some tissues. However, Southern blotting demonstrated that the 251-bp product strongly hybridized with a rat UCN cDNA probe whereas other PCR products of differing sizes did not, confirming a specific amplification of UCN (see Fig. 2B).

The RPA with antisense UCN riboprobe demonstrated that the level of UCN mRNA expression was high in midbrain and hypothalamus but low in hippocampus, pituitary, and muscle and was undetectable in adrenal, fat, and heart (Fig. 3). A typical result from the initial RPA using either sense or antisense UCN riboprobes and tissue from restrained (R) or non-stressed (C) rats is shown in Fig. 4. Sense UCN mRNA was detected with the antisense probe in midbrain and hypothalamus but not heart or liver. Antisense UCN RNA was detected by the sense probe in heart and at low levels in the hypothalamus but not in midbrain or liver. Basal levels of antisense RNA expression in heart was comparable to that of UCN sense mRNA in midbrain. Contrary to the results from our pilot study (Fig. 1) 1 h of restraint did not cause a statistically significant increase in the level of sense UCN mRNA expression in either the hypothalamus or midbrain. The level of expression of antisense UCN, compared with β-actin, was increased by 1 h of restraint stress (see Fig. 5). The presence of antisense RNA was confirmed in a second RPA using the same sense riboprobe with various tissues and 10 pg of *in vitro* transcribed UCN antisense RNA as a control. High levels of antisense RNA were detected in heart and muscle, weak expression in hypothalamus, pituitary, and cortex and no expression in any other tissue tested (Fig. 6), suggesting a tissue selective distribution of UCN antisense RNA. The hybrid for control antisense UCN RNA was 20 bp larger than that for tissue samples due to hybridization of vector sequences.

Two additional sense UCN riboprobes, spanning a 280-bp region of the 5′ end and a 258-bp region of the 3′ end of the rUCN 579-bp sequence, were used in the RPA to further characterize the sequence of the antisense transcript. All three probes detected protected signals in heart and muscle but not in midbrain (Fig. 7). As the RPA is intolerant of mismatched bases, these results demonstrate that the sequences of sense and antisense transcripts of rUCN are identical from base 1 to 560. The results of the Northern blot (see Fig. 8) demonstrate that sense and anti-sense mRNA hybridize to RNA transcripts of identical size somewhat greater than 500 bp, implying the majority of the sequence of the antisense rUCN transcript has been confirmed by the RPA.

Strand-specific RT-PCR was used to determine whether the apparent differential tissue distribution of UCN sense and anti-

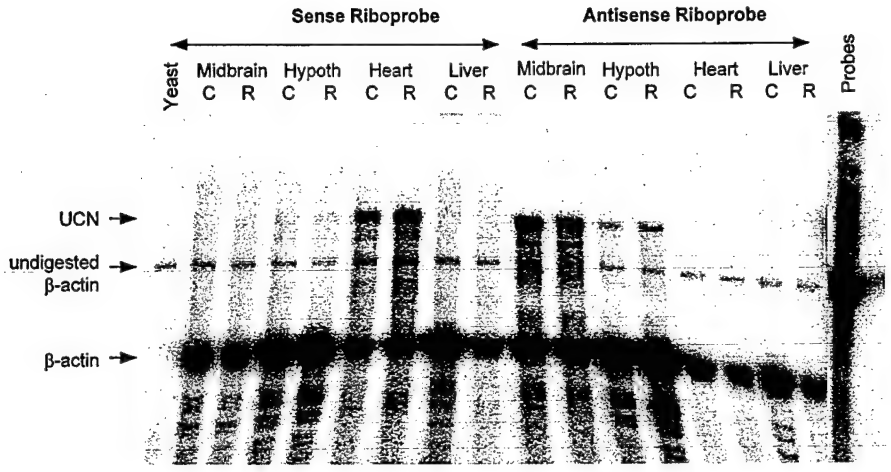

FIG. 4. Ribonuclease protection assay of 20 μg of total RNA from different tissues of control (C) or restrained (R) rats using either sense or antisense riboprobes corresponding to bases 153 to 403 of urocortin (UCN). Protected fragments of both UCN and β-actin are indicated by arrows.

sense RNA, was due to insensitivity of the RPA assay. The expected 292-bp PCR product was seen after 30 or 35 cycles of PCR in positive RT reactions but was not detected in reactions that did not include reverse transcriptase (see Fig. 7). All tissues tested expressed both sense and antisense RNA. Although RT-PCR is not quantitative, the relative abundance of amplification products was consistent with the results of the RPA, which indicated that UCN sense mRNA was expressed at higher levels in midbrain than other tissues, whereas UCN antisense RNA was more abundant in heart and skeletal muscle.

DISCUSSION

The studies described here demonstrate UCN mRNA expression in both central and peripheral tissues. High levels of sense UCN mRNA expression were detected in midbrain, consistent with findings from *in situ* hybridization and immunohistochemistry studies reported by others [6,29]. We detected a low level of UCN mRNA expression in the pituitary gland, in contrast to reports by Wong et al. [29] of high levels of hybridization in the intermediate and anterior lobes of the rat pituitary and of Bittencourt et al. [6] who were unable to detect UCN mRNA expression or UCN protein in rat pituitary cells. Iino et al. [8] reported that both UCN mRNA and protein were present in the human anterior pituitary gland but that neither were detectable in the hypothalamus or pituitary stalk [9], suggesting that UCN does not influence the hypothalamic-pituitary-adrenal axis. The discrepancy in results from hybridization and immunohistochemical studies may reflect differences in reagent specificity or stringency of hybridization conditions, however, further studies are needed to clarify differences in Ucn expression between species.

It is known that, in rats, CRF mRNA is expressed in both the central nervous system and peripheral tissues [3,17,24]. Thymus and spleen secrete immunoreactive CRF which has been implicated in local regulation of the immune response, by either paracrine or autocrine action [3]. The physiological relevance of peripheral expression of UCN mRNA remains to be fully determined although it has been reported that UCN inhibits the tumor necrosis factor-α response to an endotoxic stress [2] and that it may protect cardiac myocytes against apoptosis induced by hypoxia [19]. It has recently been reported that UCN mRNA

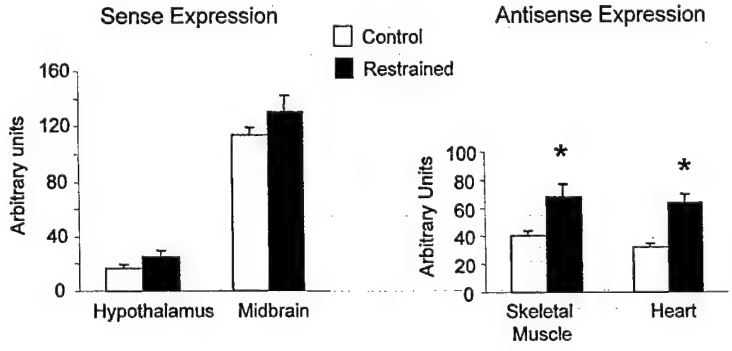

FIG. 5. Effect of restraint stress on sense and antisense urocortin (UCN) RNA expression in midbrain, hypothalamus, heart, and skeletal muscle. Expression is presented as the ratio of UCN to β-actin and is the mean + SEM for four or five rats. An asterisk indicates a significant difference ($p < 0.05$) between control and restrained rats.

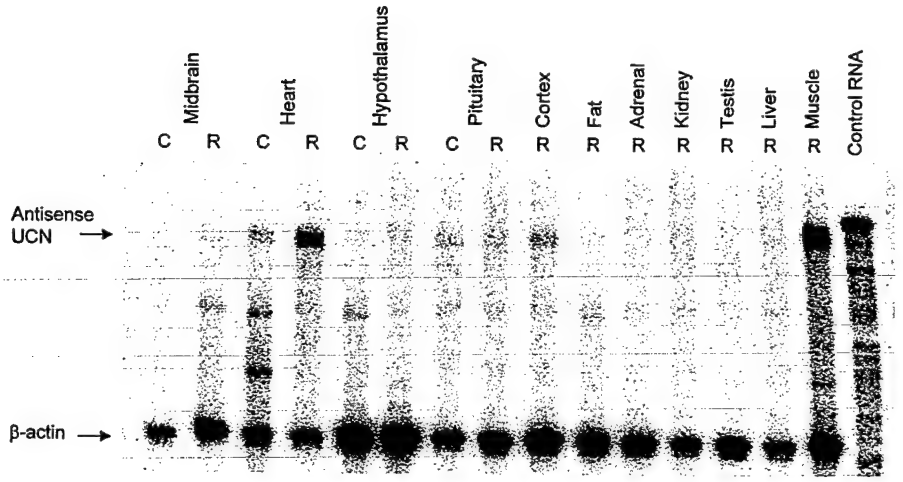

FIG. 6. Expression of antisense urocortin (UCN) RNA, detected in an ribonuclease protection assay using a sense UCN riboprobe corresponding to bases 153 to 403 of UCN. Protected fragments of antisense UCN and β-actin are indicated by arrowheads. 10 pg of *in vitro* transcribed antisense RNA was used as an control. Abbreviations: C, control rats; R, restrained rats.

is abundant in the gastrointestinal tract and is expressed in the enteric nervous system [7], suggesting a role in the regulation of intestinal motor function. UCN expression has also been detected in the thymus and spleen, where it was stimulated following an endotoxic challenge of the immune system [10]. The increase in expression was found to be corticosterone-dependant [10], whereas hypothalamic CRF and hypothalamic-pituitary-adrenal axis activity are inhibited by corticosterone [21]. These observations imply differential regulation of the two stress peptides and that UCN may be responsible for sustaining specific aspects of a stress response.

The existence of naturally occurring antisense RNAs has been demonstrated in a number of eukaryote systems [11,13, 26], including antisense expression of pro-melanin-concentrating hormone in the human hypothalamus [14] and gonadotropin-releasing hormone in the rat hypothalamus [1]. Our experiments demonstrate natural UCN antisense transcripts in rat tissue which appear to have a sequence complementary to the 579-bp sequence of sense UCN mRNA described in Genebank (U33935). The size of the transcript detected by Northern blot in our experiment was greater than 500 bp but less than 1000 bp, consistent with the approximately 1000 bp for rat UCN mRNA reported by Vaughan et al. [27] and 900 bp reported for mouse UCN mRNA by Weninger et al. [28]. It is possible that the sense and antisense RNAs are transcribed from opposite strands of the same UCN locus (cis-encoded antisense) and display perfect complementarity. The regulation of gene expression by endogenous antisense RNAs seems of general

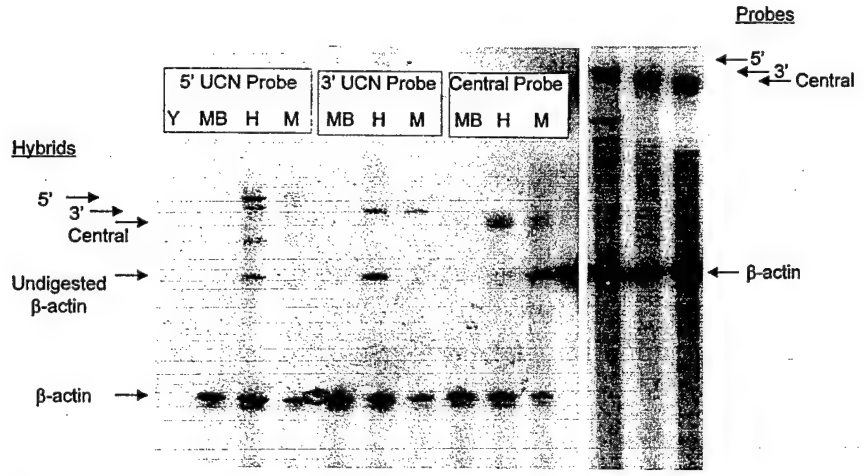

FIG. 7. Detection of antisense urocortin (UCN) RNA in an ribonuclease protection assay using 20 μg of total RNA from tissues from restrained rats and three different sense UCN riboprobes, spanning almost the full length of UCN (bases 1 to 560). Protected fragments of antisense UCN and of β-actin are indicated by arrows. Abbreviations: H, heart; Y, yeast; M, skeletal muscle; MB, midbrain.

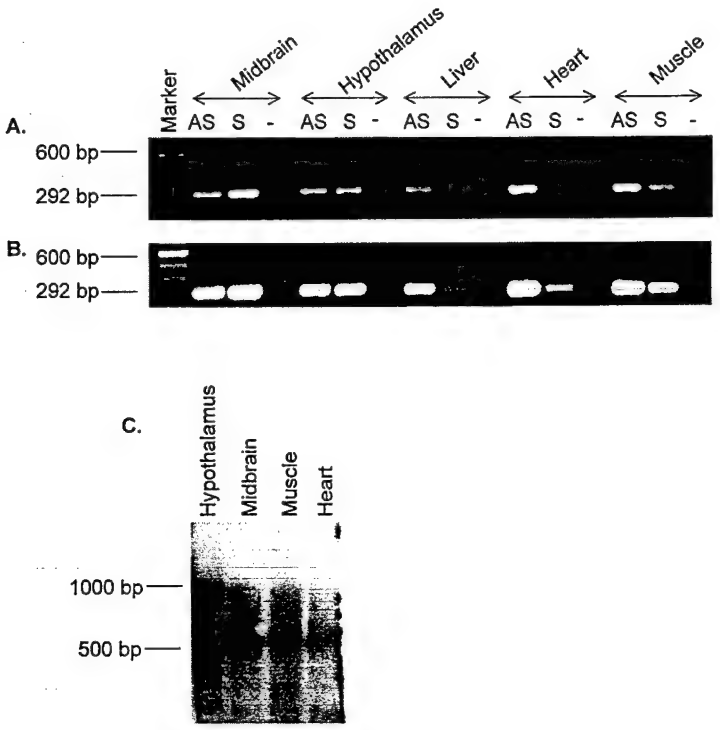

FIG. 8. Panels A and B: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of urocortin (UCN) sense and antisense RNA in rat tissues, using strand specific oligonucleotide RT primers. PCR reactions utilized primers rUCN-P4/P5. The sense and antisense 292 bp product was observed all tissues: (A) 30 cycles of PCR. (B) 35 cycles of PCR. Abbreviations: AS, antisense: RT reactions primed with rUCN-P1, which binds to antisense RNA of the UCN gene: S, sense: RT reaction primed with rUCN-P3, which binds to sense RNA of the UCN gene; −, reactions performed without RT. Panel C: Northern blot using a cDNA probe for the 3′ region of sense UCN. The midbrain contains high concentrations of sense UCN mRNA, whereas heart and skeletal muscle contain high concentrations of antisense UCN. Sense and antisense transcripts are of similar size (sense UCN = 579 bp).

importance in eukaryotes and is established in prokaryotes [11]. An open reading frame was not identified in UCN antisense RNA sequence, implying that it was not translated. There is evidence for coupled, balanced, or unbalanced expression of sense and antisense RNAs in other systems, suggesting that antisense transcripts govern the expression of their sense counterparts [11,26]. This control can be exerted at many levels of gene expression (transcription, maturation, transport, stability, and translation) and the importance of antisense UCN in regulating UCN gene expression and function remains to be determined. The PCR data showed that sense and antisense UCN transcripts were coexpressed in most tissues. The RPA provided a less sensitive but quantitative method of detecting UCN sense and antisense expression. Based on this assay, a significant amount of sense mRNA was expressed in midbrain, the hypothalamus had low levels of expression of both sense and antisense transcripts, whereas antisense transcripts were abundant in heart and muscle. The coexistence of UCN sense and antisense transcripts within the same tissue and stimulation of antisense expression by stress supports the concept of antisense UCN regulating sense UCN, possibly by inhibition of transcription. Alternatively, a number of studies have demonstrated RNA duplex formation *in vivo* [19] which would allow UCN antisense RNA to modify, destabilize, or down-regulate expression of sense UCN mRNA. If antisense UCN inhibits transcription or translation of sense UCN, the results described here suggest that UCN production during stress would be totally abolished in the heart and skeletal muscle and at least partially inhibited in the hypothalamus.

In summary, UCN antisense transcripts have been identified in central and peripheral rat tissues, with significant levels of expression in both cardiac and skeletal muscle. UCN antisense RNA is coexpressed with sense mRNA in many tissues and both are responsive to restraint stress, suggesting that the UCN antisense transcript is involved in regulating UCN gene expression in both basal and stressful conditions. Expression of antisense UCN was detectable only by RT-PCR in all tissues tested except the hypothalamus and muscle, which had levels of expression high enough to be detected by RPA. This suggests that, if antisense UCN mRNA plays a role in regulating sense UCN expression, it does so in a tissue specific manner.

ACKNOWLEDGEMENT

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Chronic Weight Loss In Rats Exposed To Repeated Acute Restraint Stress Is Independent Of Energy Balance or Leptin Status.

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ABSTRACT

Acute release of CRF during repeated restraint (3-hrs restraint on each of 3 days) causes temporary hypophagia but chronic suppression of body weight in rats. Here we demonstrated that a second bout of repeated restraint caused additional weight loss but continuing restraint daily for 10 days did not increase weight loss because the rats adapted to the stress. In these two studies serum leptin, which suppresses the endocrine response to stress, was reduced in restrained rats. Peripheral infusion of leptin before and during restraint did not prevent stress-induced weight loss, although stress-induced corticosterone release was suppressed. Restrained rats were hyperthermic during restraint but there was no evidence that fever or elevated free IL-6 caused the sustained reduction in weight. Restraining food restricted rats caused a small, but significant, weight loss. Food restricted rats fed ad libitum after the end of restraint showed a blunted hyperphagia and slower rate of weight regain than their controls. These results indicate that repeated acute stress induces a chronic change in weight that is independent of stress-induced hypophagia and may represent a change in homeostasis that is initiated by repeated acute activation of the central CRF system.

Key Words: CRF, food intake, food restriction, leptin, corticosterone

INTRODUCTION

Rats exposed to repeated restraint stress experience a temporary reduction in 24 hour food intake but maintain a reduced body weight, compared with controls (9). The sustained weight loss is dependent upon the acute central release of corticotrophin releasing factor (CRF) (24) but there is no sustained activation of this pathway to account for the maintained suppression of body weight (23). CRF activates the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system, serotonergic and catecholamine systems. All of these systems have the potential to inhibit food intake and reduce body weight but none of them are activated significantly during the hours, or days, following exposure to repeated restraint (9, 23).

All of the weight loss that occurs on the days of restraint is accounted for by a loss of lean tissue. During the days immediately following the end of repeated restraint, there is a shift in tissue metabolism so that the difference in weight between control and restrained rats is a combination of both lean and fat tissue (26). The reduction in weight is relatively small and represents only 5 to 10% of body weight (9) but there are aspects of the model that are unique. First, the stressed rats do not make any attempt to overeat once stress is ended, implying that the feedback signals that promote hyperphagia following weight loss due to food restriction (7) are not responding to the loss of weight caused by stress. Second, the maintenance of a reduced body weight for extended periods of time provides a model that can be used to examine mechanisms that normally regulate body weight. Although the stressed rats gain weight once stress has ended they do not recover the weight that has been lost during the period of restraint and remain significantly lighter than their controls (9). Weight changes induced by environmental conditions such as diet composition (12), food restriction (7) or temperature (18) are usually reversed as soon as the environmental factor is removed. A sustained reduction of

body weight in ad libitum fed animals is found in rats with lesions of the lateral hypothalamus (14) or mice in which gene expression has been modified (13) but this stress model provides an opportunity to examine chronic weight reduction in an intact animal.

As mentioned above, we have demonstrated that the prolonged reduction in body weight of rats exposed to repeated restraint can be blocked by infusing a CRF receptor antagonist into the third ventricle immediately before each stress (24), but have been unable to demonstrate any sustained activation of the CRF system during the post-stress period (9, 23). Thus the mechanism of the response has not been elucidated but it appears that the repeated acute release of CRF initiates a cascade of events that resets the homeostatic equilibrium of the animal, with a reduced body weight being a representative end point of the new equilibrium. In order to have a better understanding of the limits of the response it was necessary to clarify whether the amount of weight that could be lost during stress was limited, or was proportional to the number of exposures to stress. In addition, others have shown that weight loss in rats injected with turpentine to induce as inflammatory response correlates with the energy status of the animal (16, 17). Overfed animals lose more weight and overfed animals lose less weight than ad libitum fed rats. Therefore, the objective of the first two experiments described here was to test whether weight loss in restrained rats was proportional to the number of episodes of stress they experienced. In the process of conducting these studies we found that leptin was unexpectedly low in restrained rats. Heiman et al (11) have reported that leptin, a cytokine released from adipose tissue that may be a negative feedback signal in the regulation of energy balance (25), suppresses the endocrine response to stress. Additionally, it has been shown that leptin treatment of rats or mice specifically reduces body fat but protects muscle mass (1). All of the initial weight loss in restrained rats is lean body mass (26), therefore, the third experiment tested

whether chronic treatment with leptin would prevent stress-induced weight loss or change the composition of the loss. Finally, in order to determine whether the weight loss of restrained rats was dependent upon the energy status of the rats at the time of stress, we tested whether restraint stress inhibited food intake or body weight of rats that were already food restricted and weight reduced.

METHODS

Experiment1: *Ten days of repeated restraint*

In previous experiments rats were exposed to 3 hours of restraint stress on each of three consecutive days and this resulted in a chronic down-regulation of body weight (9). In this experiment we determined whether additional days of restraint would produce a progressively greater weight loss in the rats.

Twelve Wistar male rats (Harlan Sprague Dawley, IN), weighing approximately 400 g, were housed individually in hanging wire mesh cages with free access to food and water in a room maintained at 23 $\pm$ 2 °C, with lights on 12 hours a day from 6.30 a.m. They were adapted for 10 days to a diet containing 40% kcal fat, 16% kcal protein, because a high fat diet exaggerates the energy balance response to restraint stress (9). Baseline daily food intakes and body weights were recorded for 7 days and then the rats were divided into two weight-matched groups. One was exposed to 3 hours of restraint each morning, from 7.00 to 10.00 a.m., on each of ten consecutive days. The non-stressed controls were moved to the same room as restrained rats and housed without food or water for the period of restraint. On Days 1, 3, 5, 7 and 9 of restraint a small blood sample was collected from the tail of each rat for measurement of corticosterone (Corticosterone RIA kit: ICN Diagnostics, CA) and leptin (Rat Leptin RIA kit: Linco Research Inc., MO). On days 1, 4, 6 and 10 of restraint rectal temperatures were

measured at the end of restraint. Two and five days after the end of restraint a tail blood sample was collected at 10.00 a.m., following a 3 hour fast, for measurement of corticosterone and leptin. Twelve days after the end of restraint the rats were decapitated between 9.30 a.m. and 10.30 a.m. following a 3 hour fast. Trunk blood was collected for measurement of corticosterone and leptin and the carcass, less gut content, was analyzed for composition (6).

Experiment 2*: Two bouts of 3 days of repeated restraint*

This experiment tested whether weight loss induced during the first bout of repeated restraint represented a maximal response to the stimulus or whether exposure to a second bout of repeated restraint caused additional weight loss in the rats. Serum IL-6 and rectal temperatures were also measured, to test whether repeated restraint induced an inflammatory response.

Fourteen male Wistar rats, weighing approximately 400 g, were housed in individual cages with free access to water and the 40% kcal fat diet. After 10 days they were divided into two weight-matched groups and one was exposed to 3 hours of restraint on each of 3 days. On the morning before the start of stress, and for the next 5 days, a small tail blood sample was collected at a time that was equivalent to 1 hour after the end of restraint. Serum leptin and free IL-6 (Quantikine M IL-6: R&D Systems, MN) were measured. Rectal temperatures of the rats were measured immediately before and immediately after restraint and at an equivalent time on the four days following stress. Seven days after the end of restraint the rats were exposed to a second bout of repeated restraint. Rectal temperatures were measured and blood samples were collected as before. The experiment ended on Day 26, thirteen days after the end of the second bout of repeated restraint and carcass composition was determined (6).

Experiment 3: *The Effect of Chronic Leptin Infusion in Rats Exposed to Repeated Restraint*

Experiments 1 and 2 indicated that leptin declined during the days following repeated restraint. It is well established that leptin has little effect on lean mass while reducing body fat (1, 4, 5), and it has been reported that leptin inhibits some of the HPA responses to stress (11). Therefore, we tested whether chronic infusion of leptin before and during repeated restraint would prevent weight loss during restraint, which is predominantly lean tissue (26), or influence the body composition of the rats during the post-stress period.

Forty-eight male rats, weighing 375 g, were housed individually with free access to water and the 40% kcal fat diet. Food intakes and body weights were recorded daily for 10 days after which the rats were divided into three weight-matched groups. Each rat was anaesthetized with isofluorane and an Alzet miniosmotic pump (Model 2 ML4, Alza Corporation, Newark, DL) delivering 0, 30 or 100 ug/day recombinant rat leptin (R&D Systems) for 28 days was placed in the intraperitoneal cavity. Two days after pump placement a small tail blood sample was collected in the morning after a 3 hour fast to measure serum leptin and corticosterone.

On Day 21 of leptin infusion the rats within each leptin treatment group were subdivided into two weight-matched groups. One subgroup was exposed to 3 hours of restraint on each of 3 days and the other subgroup was a non-restrained control. One hour after the start of restraint on the first day of stress a tail blood sample was collected for measurement of leptin and corticosterone. On the day after the end of stress the rats were food deprived for 3 hours in their home cages and a blood sample was collected for measurement of leptin and corticosterone. Five days after the end of restraint the rats were decapitated. Trunk blood was collected for measurement of serum corticosterone, leptin, insulin, glucose and free fatty acids (NEFA C kit: WAKO Chemicals, TX). Epididymal fat was weighed, snap frozen and total RNA was extracted for measurement of leptin mRNA expression by Northern blot (8). Thymus, adrenal glands, liver

and inguinal, retroperitoneal (RP), perirenal (PR) and mesenteric fat pads were dissected and weighed before being returned to the carcass. The carcass, less gut content, was analyzed for composition (6).

Experiment 4: *Repeated restraint in weight reduced, food-restricted rats.*

In other experimental protocols that induce a prolonged down-regulation of body weight, the energy balance status of the animal at the time that weight loss is induced determines the amount of weight that is lost. For example, weight loss caused by an inflammatory response is exaggerated if the rats have been overfed before being exposed to turpentine, whereas they gain weight if they are weight-reduced at the time of treatment (17). The objective of this experiment was to determine whether weight-reduced rats would lose weight in response to repeated restraint stress.

Sixty male Wistar rats, weighing approximately 320 g, were single housed in suspended stainless steel cages with ad libitum access to water and the 40% kcal fat diet. Food intakes and body weights were recorded daily for a week before the rats were divided into three weight-matched groups. One group had ad libitum access to food and the two remaining groups were restricted to 50% of their voluntary food intake for 10 days, by which time food restricted rats weighed 75 g less than ad libitum fed animals. Food restricted rats were given their food at the onset of the dark cycle.

Each of the three groups was divided into two weight-matched subgroups. One subgroup was exposed to repeated restraint and the other was a non-stressed control group. A small blood sample was collected from each rat by tail bleeding after the first hour of restraint on the first day of stress for measurement of serum corticosterone. After the third restraint, the ad libitum group continued to eat ad libitum (AL), one group of food restricted rats remained on 50% voluntary

food intake (FR) and the rats in the second food restricted group were returned to ad libitum feeding (FR-AL). Twelve days after the end of restraint the rats were killed in the morning, between 9.00 and 11.00 a.m. Trunk blood was collected for determination of serum corticosterone, leptin, glucose, insulin, and free fatty acids. The carcass, less gut content, was analyzed for composition

Statistical Analysis

Daily body weight and food intake measures were modeled separately with repeated measures ANOVA. Initial body weight or food intake at the start of stress was used as a covariate in the analysis. Post-hoc comparisons of treatment means at different time points were effected by t-test and the significance levels reported are unadjusted for multiple comparisons (SAS for Windows, Release 6.12: SAS Institute, NC). Differences in single time point measures were determined by t-test, one-way ANOVA or two-way ANOVA and post-hoc Duncans Multiple range test (Statistica: StatSoft, Tulsa, OK).

RESULTS

Experiment 1: Restraint Stress on Ten Consecutive Days

Exposing rats to repeated restraint for 10 days caused a significant loss of body weight (Figure 1A: Restraint: $P<0.002$, Day: $P<0.0001$, Int: $P<0.0001$), such that restrained rats weighed significantly less than controls from Day 3 to the end of the experiment. The extended period of repeated restraint did not increase the amount of weight lost because restrained rats weighed significantly less on Day 5 of restraint than Day 0, but their body weight was not significantly different on Day 10 of restraint compared with Day 3. Stress inhibited food intake on the days of restraint, but the intake of restrained rats returned to control levels once stress ended (Figure 1B: Restraint: $P<0.02$, Day: $P<0.0001$, Int: $P<0.0001$). Restraint stress caused a

significant increase in serum corticosterone (Figure 2A: Restraint: $P<0.03$, Day: $P<0.0001$, Int: $P<0.0001$) and body temperature (Figure 2B: Restraint: $P<0.04$, Day: $P<0.0009$, Int: $P<0.004$). The rats appeared to be adapting to repeated exposure to stress because corticosterone was significantly higher following restraint on Day 1 than on Days 7 or 9. Similarly, rectal temperatures were higher on Days 1, 4 and 6 than on Day 10. Temperatures of the controls were also elevated on Day 1, which suggests that being moved to a novel environment was stressful. In contrast, repeated restraint caused a delayed decline in serum leptin (Figure 2C: Restraint: $P<0.08$, Day: $P<0.005$, Int: $P<0.0005$). Leptin in restrained rats was significantly lower than that in controls from Day 7 of restraint to Day 17 of the experiment, a week after the stress had ended. The reduced body weight of restrained rats was accounted for by non-significant decreases in both fat and lean tissue (Control Fat: 71 ± 11 g, Restrained Fat: 52 ± 3g. Control Lean Mass 389 ± 7 g, Restrained Lean Mass: 378 ± 7 g).

Experiment 2: Two Bouts of Repeated Restraint

Exposing rats to two bouts of repeated restraint had an additive effect on weight loss. Restrained rats weighed significantly less than controls from the last day of the first bout of repeated restraint (Day 3) to the end of the experiment (Figure 3A: Restraint: $P<0.0001$, Day: $P<0.0001$, Int: NS). The difference in weights between the two groups plateaued after the first bout of restraint and was then increased by the second bout of restraint. This response is illustrated in Figure 3B, which is a plot of the difference between the mean body weight of the two groups of rats on each day of the experiment. Restraint significantly inhibited food intake (Figure 3C: Restraint: $P<0.04$, Day: $P<0.0001$, Int: NS), but only on the days that stress was applied. Restraint caused a significant elevation of rectal temperature, compared with either controls or with pre-stress temperatures (Figure 4: Restraint: $P<0.0001$, Day: $P<0.001$, Pre-/Post-

stress: P<0.0001, Restraint x Day: P<0.0001, Pre-/Post-stress x Restraint: P<0.0001, Pre-/Post-stress x Day: P<0.0001). Hyperthermia was transient and was not present 24 hours after the end of stress.

Serum concentrations of free IL-6 were undetectable on Day 0, before stress, in all rats, as shown in Figure 5A. After stress, free IL-6 was significantly increased in both control and restrained animals, with no differences between the two groups (Restraint: NS, Day: P<0.0001, Int: NS), indicating that the daily manipulations involved in this protocol, independent of restraint, were stressful enough to induce release of this cytokine. There were no differences in serum leptin concentrations of control and restrained rats during the first bout of restraint (Figure 5B). During the second bout of restraint leptin was lower in restrained than control rats (Figure 5C: Restraint: NS, Day: P<0.0001, Int: P<0.002) but post hoc analysis did not show any specific day on which there was a significant difference between the two groups. Carcass analysis did not show any change in the carcass fat content of the rats as all of the weight difference was accounted for as lean mass (water + protein) (Control Fat: 69 $\pm$ 2 g, Restrained Fat: 69 $\pm$ 5g. Control Lean Mass 455 $\pm$ 11 g, Restrained Lean Mass: 430 $\pm$ 12 g).

Experiment 3: Repeated Restraint of Leptin Infused Rats

There was no effect of peripheral leptin infusion on food intake or body weight during the 21 days before restraint stress in rats consuming a 40% kcal fat diet, as shown in Table 1. Serum leptin concentrations, measured 2 days after the start of infusion, were increased approximately 4-fold in rats receiving 100 ug/day, compared with controls (Table 1). There was no effect of leptin on serum corticosterone, glucose or FFA at this time point. By the end of the study serum leptin was about 7-fold higher in the 100 ug/day group than in controls (Figure 7A). All of the rats that were exposed to repeated restraint stress lost weight but rats infused with 100

ug/day leptin appeared to regain weight faster than the two other groups (Figure 6A: Leptin: NS, Stress: $P<0.03$, Day: $P<0.0001$, Stress x Day: $P<0.002$, Leptin x Day: $P<0.02$). All rats exposed to repeated restraint ate less during stress than their controls. There was no effect of leptin on this response (Figure 6B: Leptin: NS, Stress: $P<0.002$, Stress x Leptin: NS, Day: $P<0.0001$, Stress x Day: $P<0.009$) and intakes returned to control levels once restraint ended. Serum corticosterone measured during the first restraint was lower in both control and restrained rats receiving 100 ug/day leptin than in comparable treatment groups receiving 0 or 30 ug/day leptin (Figure 7B: Leptin $P<0.005$, Stress: $P<0.06$, Int: NS). There were no differences in corticosterone measured the day after the end of repeated restraint (Figure 7B) but at the end of the experiment, 5 days after the end of restraint, corticosterone was lower in all rats that had been restrained than in controls (Figure 8A: Leptin: NS, Stress: $P<0.01$, Int: NS). There were no differences in serum glucose or insulin at this time point (data not shown). Stress had no effect on serum leptin concentrations at the end of the experiment (Figure 8B) but there was a significant interaction between leptin and stress ($P<0.05$) on leptin mRNA expression in epididymal fat (Figure 8C). Expression was inhibited in restrained 100 ug/day rats, compared with their controls, even though there were no differences in serum leptin concentrations of the two groups.

Carcass weights of restrained rats were lower than those of controls (Leptin, NS, Stress $P<0.009$, Int: NS), and this difference was a combination of lean and fat tissue for all treatment groups (data not shown). The 100 ug leptin/day control group of rats was non-significantly fatter than the other animals and there were significant increases in epididymal and retroperitoneal fat pad weights (Epididymal: 100 ug/d controls 9.2 ± 0.5 g, All other groups: 7.2 - 7.8 ± 0.5 g, $P<0.05$. Retroperitoneal: 100 ug/d controls 4.8 ± 0.5 g, All other groups: 3.4 - 3.8 ± 0.3 g,

P<0.05). There were no differences in adrenal gland weight but stress significantly reduced thymus weight (Leptin: NS, Stress: P<0.01, Int: NS) especially in the 100 ug/day leptin group (Control: 376 $\pm$ 35 mg, Restrained 285 $\pm$ 21 mg, P<0.02).

Experiment 4: Restraint of Food Restricted Rats

Restraint stress caused a significant reduction in body weight, independent of the rat's feeding status (Figure 9A: Feeding Status: P<0.0001, Restraint: P<0.0001, Day: P<0.0001, Feeding Status x Restraint: P< 0.0008, Feeding Status x Restraint x Day: P<0.01). Restrained AL and FR-AL rats weighed significantly less than their respective non-stressed controls on all post-stress days. Restrained FR rats weighed significantly less than non-stressed FR rats for the last 5 days of the recovery period. Restrained rats gained less weight than their non-stressed controls during the 12 day post-stress period (Days 3 to 14), independent of feeding status (Figure 10A: Feeding Status: P<0.0001, Restraint: P<0.0001, Int: NS).

Both groups of restrained rats that had free access to food during the post-stress period ate significantly less than their control group (Figure 9B: Restraint: P< 0.001, Feeding Status: P<0.0001, Day: P< 0.003, Feeding Status x Restraint: P< 0.0001, Feeding Status x Restraint x Day: P<0.003). Restrained and control FR-AL rats were initially hyperphagic when they returned to ad libitum feeding but this was reversed earlier in the restrained than the control animals. The effect of restraint on food intake was also apparent when cumulative food intake between Days 3 and 14 of the experiment was compared for all groups of animals (Figure 10B: Feeding Status: P<0.0001, Restraint: P<0.001, Int: P<0.07).

Serum corticosterone was significantly increased during restraint stress in all rats (Figure 10C: Restraint: P<0.001, Feeding Status:NS, Int: NS) but the response was greater in FR than AL rats. Twelve days after the end of repeated restraint serum corticosterone was higher in all

groups of rats that had been restrained than in their respective control group (Table 2: Restraint: $P<0.05$, Feeding Status: NS, Int: NS). The effect was only significant in the FR animals. FFA were lower in FR and FR-AL rats than AL rats but there was no effect of restraint (Restraint: NS, Feeding Status: $P<0.0001$, Int: NS). Similarly, serum insulin concentrations were lower in FR rats than the two other groups of animals (Restraint: NS, Feeding Status: $P<0.0002$, Int: NS). Serum glucose and leptin levels measured at the end of the experiment were significantly reduced by both restraint and food restriction (Restraint: $P<0.01$, Feeding Status: $P<0.0001$, Int: NS). Glucose was lower in FR rats than the two other groups and tended to be lower in restrained than control rats in all three treatment groups but the difference was only significant for AL rats. Leptin was substantially reduced in both control and restrained FR rats compared with the other animals and in both AL and AL-FR rats serum leptin concentrations were lower in restrained than control rats.

Carcass weight, carcass fat and lean mass (protein + water) were all significantly reduced by restraint stress (Table 2: Restraint: $P<0.0001$, Feeding Status: $P<0.04$, Int: NS) but post-hoc analysis did not detect any significant differences between restrained and control rats within any of the feeding treatments. The effect of feeding status was significant for all aspects of body composition except for carcass ash.

DISCUSSION

Exposing rats to restraint stress causes weight loss and the stressed rats subsequently maintain a body weight that is 5 to 10% less than that of non-restrained controls for periods as long as 40 days after stress has ended (9, 23). This response is blocked by infusion of a CRF receptor antagonist, αhCRF, into the third ventricle immediately before restraint (24), suggesting that the chronic down-regulation of body weight is initiated by acute release of CRF in an area of

the brain close to the hypothalamus. Chronic weight loss is not limited to restraint stress as similar changes have been found in rats exposed to a single social defeat (21) or chronic mild stress (10). The restraint protocol, however, allows us to expose a relatively large number of animals to a standardized stressor. The experiments described here were carried out to provide further clarification of physiological conditions that influence the chronic response to repeated acute stress.

Previous experiments have shown that exposure to 3 hours of restraint on each of three days (9) causes a greater weight loss than that produced by one 3 hour period of restraint (23). The results of Experiments 1 and 2 demonstrate that exposing rats to a second bout of repeated restraint increased the amount of weight lost by stressed rats, but increasing the number of consecutive days that the rats were restrained did not exaggerate the weight loss caused by 3 days of restraint. This may be explained by the rats adapting to the stress in Experiment 1, such that stress-induced activation of central mechanisms that initiate the chronic response was ameliorated on successive days of restraint and the subsequent effect on body weight was limited. This concept is supported by the gradual decline in stress-induced corticosterone release during the 10 days of exposure to restraint stress. In contrast, when the rats were exposed to two bouts of restraint, with an interval of a week between bouts, the hyperthermic response to stress was as great during the second bout of restraint as the first, implying that the central response was also intact and induced further loss of body weight in the rats.

In both Experiment 1 and Experiment 2 we found a delayed decline in serum leptin concentrations during the post-stress period. When rats were restrained daily for 10 days, the reduction in leptin was significant after 5 days of restraint and was maintained for a week after the stress had ended. The time delay between onset of stress and the drop in leptin implies that it

did not result from direct inhibition of expression by activation of either the HPA axis or stress-induced release of catecholamines. In addition, these results do not support the concept of physiological concentrations of leptin having an inhibitory effect on corticosterone release in stressed animals (11), because both leptin and corticosterone were significantly reduced on the last 5 days of restraint. If leptin provided a tonic inhibition of the HPA axis, corticosterone release in response to stress would be expected to increase, or at least be maintained, once leptin concentrations declined. It is interesting to note that although leptin concentrations were reduced at the end of restraint and after stress had ended, there was no evidence of post-stress hyperphagia to compensate for the period of hypophagia during restraint. The failure to show compensatory overeating in restrained rats may contribute to their maintenance of a reduced body weight, compared with controls. Although leptin is hypothesized to be a circulating satiety signal (25) and, based on this hypothesis, food intake would be expected to increase when leptin levels decline, the results from these experiments clearly demonstrate that leptin is not responsible for either the inhibition of intake during stress, or for the failure to overeat once stress has ended.

We have previously reported that weight loss during restraint is exclusive to lean tissue but, during the days following the end of stress, the weight difference between control and restrained rats shifts to a combination of both lean and fat tissue (9, 26). In Experiments 1 and 2, body composition of the rats was measured almost two weeks after the end of stress, therefore it was not possible to determine whether the decline in leptin coincided with changes in body fat content of the rats. Unlike previous experiments (9, 26), or Experiment 1, all of the weight loss in animals that had been exposed to two bouts of repeated restraint was accounted for by lean

body mass. This suggests that a second exposure to stress does not promote the same cascade of metabolic events that are initiated by the first exposure to stress.

It has been established in normal mice that leptin protects lean mass while selectively reducing body fat content (1, 3). In addition, it has been suggested that leptin can inhibit activation of the HPA axis, as a protective mechanism in conditions of chronic stress (11). Therefore, in Experiment 3, we determined whether treating the rats with leptin before and during exposure to repeated restraint would moderate, or prevent, stress-induced weight loss. The rats in all of the experiments described here were fed a high-fat diet and peripheral leptin had no effect on food intake or body weight during the pre-stress infusion. Thus, the response to stress was not confounded by rats treated with different doses of leptin having different starting body weights or body compositions. The results of the experiment demonstrate that although the highest dose of leptin did inhibit stress-induced release of corticosterone during restraint, it did not influence the amount or composition of weight that was lost. Thus, a five-fold elevation of circulating concentrations of leptin was able to suppress activity of the HPA axis, whereas endogenous leptin in Experiment 1 did not appear to exert any limiting effect on corticosterone release. Although, the high dose of leptin inhibited stress-induced corticosterone release, it did not prevent stress-induced hypophagia or weight loss, confirming previous observations that the chronic response to stress is independent of glucocorticoid release (24). Five days after the end of restraint, corticosterone, measured in blood collected between 9.00 a.m. and 12.00 p.m., was lower in all of the rats that had been exposed to restraint than in their controls. As only a single measure was made it is not possible to determine whether there was a sustained inhibition of activity of the adrenal cortex, or whether stress had shifted the circadian rhythm of corticosterone release. Leptin expression was increased in the epididymal fat of control 100 ug

leptin/day rats compared with all other groups, although there were no differences in serum leptin concentrations between this group and the restrained rats that received the same dose of leptin. The increase in leptin expression reflected the significant enlargement of epididymal depots in these animals. It is possible that stress had reversed a similar degree of adiposity in the restrained 100 ug leptin/day rats and had also changed the rate of leptin clearance, as there were no differences in circulating concentrations of leptin in control and restrained rats infused with 100 ug leptin/day.

In the final experiment described here we determined whether rats that were already weight reduced would show the same chronic response to repeated restraint as ad libitum fed rats. Restrained rats that remained food restricted after the end of stress lost only a small amount of weight, suggesting that there is an innate protective mechanism that prevents an additive response to two simultaneous stressors. These results are similar to those reported by Gursoy et al (2) who found that daily immobilization stress did not reduce the body weight of food restricted rats. In Experiment 4, the restrained FR-AL rats were initially hyperphagic and gained weight once stress had ended. The rate at which they gained weight was not as rapid as that in the control FR-AL rats and food intake of restrained FR-AL rats returned to control levels before that of the FR-AL control rats. These results imply that, although the weight reduced rats did not lose weight in response to restraint, the mechanisms responsible for initiating a chronic down-regulation of body weight was intact and caused restrained FR-AL rats to gain less weight than their non-restrained counterparts during refeeding. Similar results have been reported for weight loss following lesions of the lateral hypothalamus. Rats that have been weight reduced have a lower energy expenditure (15) and lose less weight than ad libitum fed animals so that both groups of lesioned animals reach the same post-lesion weight (14). Other investigators (16, 17)

have shown that the metabolic response to acute inflammation caused by turpentine injection is also related to the energy status of the rats at the time of injection. Overfed rats become hypermetabolic, anorexic and lose weight whereas weight reduced rats given ad libitum access to food after the injection are hyperphagic and gain weight so that the post-inflammation weight is the same for all of the rats, independent of their energy status at the onset of inflammation. Thus it appears that both restraint stress and inflammation initiate a series of events that lead to the rats defending a reduced body weight. Because we did not find any evidence that restraint stress caused an independent stimulation of IL-6 or development of fever, it is not clear whether the two different types of stress cause weight loss through the same mechanisms or whether different mechanisms result in similar changes in the two models.

In summary, exposing rats to repeated restraint stress causes a chronic down-regulation of body weight that is initiated by acute activation of central CRF receptors (24). Exposing the rats to a second bout of restraint caused further weight loss, whereas extending the number of days of restraint within one bout did not, possibly because the rats adapted to the stressor. Although there was a delayed decline in serum leptin concentrations of restrained rats, infusing the rats with leptin before and during exposure to restraint did not protect against weight loss even though stress-induced corticosterone release was inhibited by high concentrations of leptin. Stress-induced weight loss is inhibited in weight reduced rats but animals that are allowed to refeed after the stress has ended gain less weight than their non-restrained controls. These results indicate that the reduced body weight is not dependent upon stress-induced hypophagia.

PERSPECTIVE

The effects of stress on body weight are determined both by the severity of the stress and by an individuals perception of the stress. In animals, mild stressors, such as tail pinch, increase

food intake (19), whereas more severe stressors, such as restraint or immobilization, inhibit food intake (20) and have long-term effects on body weight and behavior (9, 21). In humans, extreme stress, such as combat, inhibits food intake (22) but the chronic effects on food intake and body composition have not been determined. Experiments described here demonstrate that the sustained reduction in body weight caused by acute stress is not a direct result of hypophagia, but, based on the response of food restricted rats, it appears that repeated restraint changes some aspect of the metabolic equilibrium. This change is induced in weight reduced animals but is only expressed as a difference in body weight in ad libitum fed animals. Further studies are needed to identify other chronic behavioral and biochemical changes that are present in these animals. Elucidation of the mechanisms that cause this disruption of homeostasis would provide new information on the regulation of body composition and body weight and may also be valuable in developing new treatments for trauma patients.

Table 1: Pre-Stress body weights, food intakes and serum hormone concentrations in rats receiving intraperitoneal infusions of leptin.

	0 ug/day	**30 ug/day**	**100 ug/day**
Pre-infusion Weight (g)	402 ± 4	403 ± 4	402 ± 4
Pre-stress Weight (g)	430 ± 5	429 ± 5	434 ± 5
Pre-stress Food Intake (g/21 d)	300 ± 5	300 ± 5	297 ± 5
Day 2 of Leptin Infusion			
Leptin (ng/ml)	4.7 ± 0.6^{A}	6.2 ± 0.5^{A}	16.5 ± 2.6^{B}
Corticosterone (ng/ml)	19 ± 3	15 ± 1	17 ± 2
Glucose (mMol/L)	5.6 ± 0.3	5.6 ± 0.3	6.0 ± 0.3
Free Fatty Acids (uEq/L)	670 ± 33	659 ± 31	651 ± 31

Data are means ± sem for groups of 16 rats. The pre-infusion weight is the weight of the rats on the day that leptin infusions started. The pre-stress weight is the weight of the rats on the morning before the first restraint stress, after 21 days of leptin infusion. Pre-stress food intake is the total amount of food eaten during the 21 days of leptin infusion before the start of restraint stress. Values for leptin, measured after 2 days of infusion, that do not share a common superscript are significantly different, determined by one-way or two-way ANOVA.

Table 2: Serum hormones and body composition of food restricted rats exposed to repeated restraint.

	AL		FR		FR-AL	
	Control	**Restrained**	**Control**	**Restrained**	**Control**	**Restrained**
Day 12 Recovery						
Corticosterone (ng/ml)	35 ± 10^{A}	57 ± 16^{AB}	70 ± 16^{B}	153 ± 70^{B}	55 ± 12^{AB}	117 ± 35^{B}
Insulin (ng/ml)	2.6 ± 0.2^{A}	2.5 ± 0.3^{A}	1.3 ± 0.1^{C}	1.5 ± 0.2^{BC}	2.4 ± 0.2^{A}	1.8 ± 0.3^{B}
Leptin (ng/ml)	26 ± 2^{A}	19 ± 3^{B}	4 ± 0.5^{C}	4 ± 0.4^{C}	20 ± 2^{B}	14 ± 2^{D}
FFA (mEq/L)	0.60 ± 0.05^{A}	0.51 ± 0.05^{A}	0.45 ± 0.02^{B}	0.44 ± 0.04^{B}	0.39 ± 0.04^{B}	0.48 ± 0.03^{AB}
Glucose (mg/dl)	269 ± 15^{A}	231 ± 9^{B}	216 ± 10^{C}	200 ± 13^{C}	260 ± 12^{A}	241 ± 15^{AB}
Carcass Composition (g/rat)						
Weight	466 ± 16^{A}	434 ± 17^{A}	323 ± 7^{B}	314 ± 7^{B}	416 ± 13^{AC}	391 ± 12^{C}
Protein	146 ± 5^{A}	140 ± 6^{A}	98 ± 3^{BC}	95 ± 2^{C}	135 ± 6^{AB}	112 ± 9^{B}
Water	260 ± 7^{A}	249 ± 7^{A}	201 ± 4^{B}	194 ± 7^{B}	240 ± 8^{AC}	233 ± 9^{C}
Fat	47 ± 6^{A}	32 ± 6^{AC}	10 ± 5^{B}	6 ± 1^{B}	29 ± 4^{C}	26 ± 2^{C}
Ash	14 ± 0.9	13 ± 0.4	13 ± 0.7	12 ± 0.6	13 ± 0.7	13 ± 0.4

Data are means $\pm$ sem for groups of 10 rats killed 12 days after the end of repeated restraint. Values for a specific parameter that do not share a common superscript are significantly different at $P<0.05$, determined by two-way ANOVA and post-hoc Duncan's Multiple Range test.

FIGURE LEGENDS

Figure 1: Daily body weights (A) and food intakes (B) of Wistar rats exposed to 3 hours of restraint on 10 consecutive days. Data are means $\pm$ sem for groups of 6 rats. Repeated measures analysis indicated a significant difference in the weights of control and restrained rats from Day 3 to the end of the experiment, as indicated by asterisks. Food intake of restrained rats was inhibited only on days that the animals were restrained.

Figure 2: Repeated measures of corticosterone (A), rectal temperature (B) and leptin (C) of rats exposed to 3 hours of restraint on each of ten days. Data are means $\pm$ sem for groups of 6 rats. Values for control or leptin treated rats that do not share a common superscript are significantly different at $P<0.05$. Asterisks indicate significant differences between control and restrained rats.

Figure 3: Daily body weights (A), difference in average weights between stressed and control rats and daily food intakes (C) of Wistar rats exposed to two bouts of repeated restraint stress. Data are means $\pm$ sem for groups of 7 rats. Restraint caused a significant reduction in body weight, indicated by asterisks, which was apparent from day 4 to the end of the experiment. Food intake was inhibited only on days that rats were restrained.

Figure 4: Repeated measures of rectal temperatures of rats exposed to two bouts of repeated restraint on days 1 to 3 and days 10 to 12. Temperature was measured at the end of 3 hours restraint. Data are means $\pm$ sem for groups of 7 rats. Asterisks indicate that stress caused a significant elevation of body temperature at the end of 3 hours restraint.

Figure 5: Repeated measures of serum free IL-6 (A) during the first of two bouts of repeated restraint and of leptin (B and C) during both of the bouts of repeated restraint. Data are means ± sem for groups of 7 rats. The manipulations of the study caused significant elevations in IL-6 for all rats but there was no difference between control and restrained animals. There was a significant interaction between stress and time ($P<0.002$) for leptin concentrations, determined by repeated measures analysis, but post-hoc tests did not reveal a significant difference on any specific day of the experiment.

Figure 6: Change in body weight (A) and daily food intake (B) of rats infused with PBS or leptin for 21 days before being exposed to repeated restraint. Data are means ± sem for groups of 8 rats. Stress caused a significant weight loss in all groups of rats, as indicated by an asterisk. On the last two days of the experiment the difference in weight was significant only for the 0 and 30 ug leptin/day groups, as indicated by #. Food intakes of the rats were inhibited by restraint. This difference was significant for all three groups on days 22 and 24 (*) but only for the 0 and 100 ug leptin /day groups on day 23 (ψ).

Figure 7: Serum leptin (A) and corticosterone (B) concentrations in rats that were infused with leptin before and during restraint, measured after 1 hour of restraint on the first day of repeated restraint or at an equivalent time one day after the end of repeated restraint. Data are means ± sem for groups of 8 rats. Superscripts indicate significant difference between group within each time point.

Figure 8: Serum corticosterone (A), serum leptin (B) and adipose leptin mRNA expression (C) measured 8 days after the end of repeated restraint in rats infused with increasing concentrations of leptin before and during restraint. Data are means $\pm$ sem for groups of 8 rats. Superscripts indicate differences between in serum leptin concentration and leptin expression ($P<0.05$).

Figure 9: Daily body weight (A) and food intakes (B) of rats that were fed ad libitum (AL) or were food restricted (FR) throughout the experiment or were food restricted to 50% voluntary intake for 10 days before the experiment and returned to as libitum feeding at the end of restraint (FR-AL). Data are means $\pm$ sem for groups of 10 rats. An asterisk indicates a significant difference between restrained rats and their controls, determined by repeated measures analysis of variance and post-hoc t-test.

Figure 10: Weight change (A) and food intake (B) of AL, AL-FR and FR rats during 12 days that followed the end of repeated restraint. Data are means $\pm$ sem for groups of 10 rats. Panel C represents serum corticosterone concentrations measured after 1 hour of restraint on the first day of repeated restraint in AL (n=10) and FR rats (n=20). Values for a specific parameter that do not share a common superscript are significantly different at $P<0.05$.

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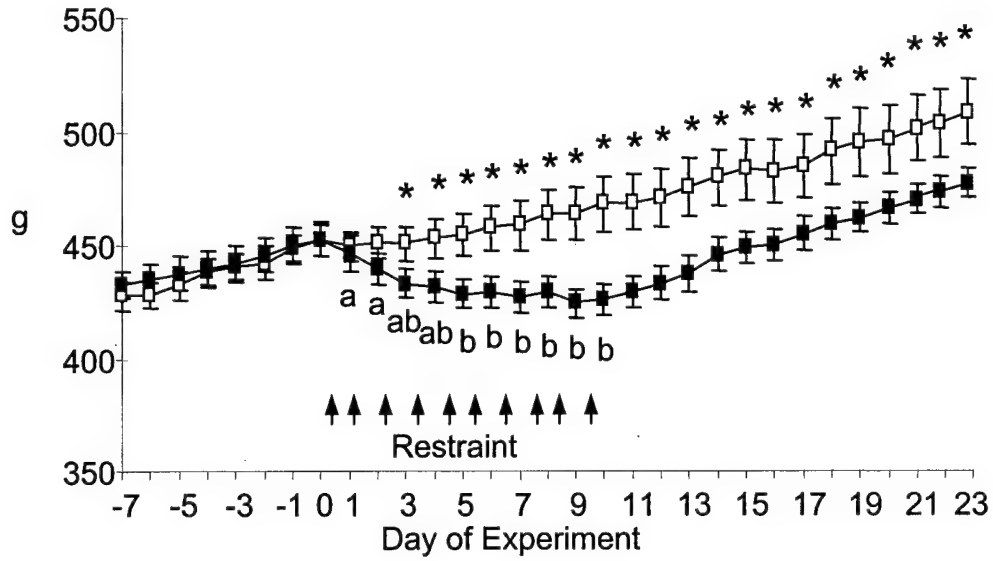
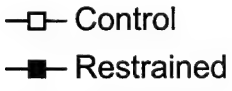
Figure 1:

A: Body Weight of Rats Restrained on 10 Consecutive Days





B: Food Intake of Rats Restrained on 10 Consecutive Days

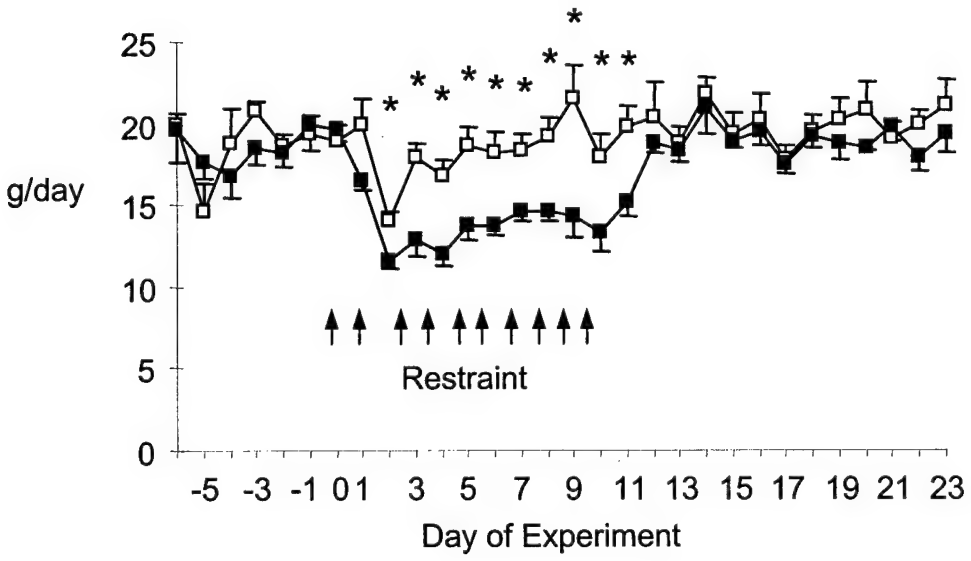
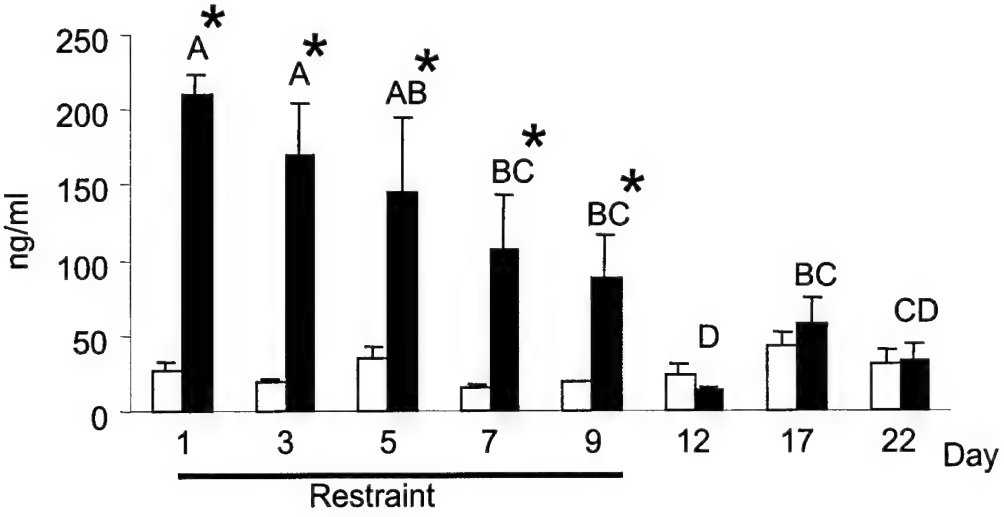
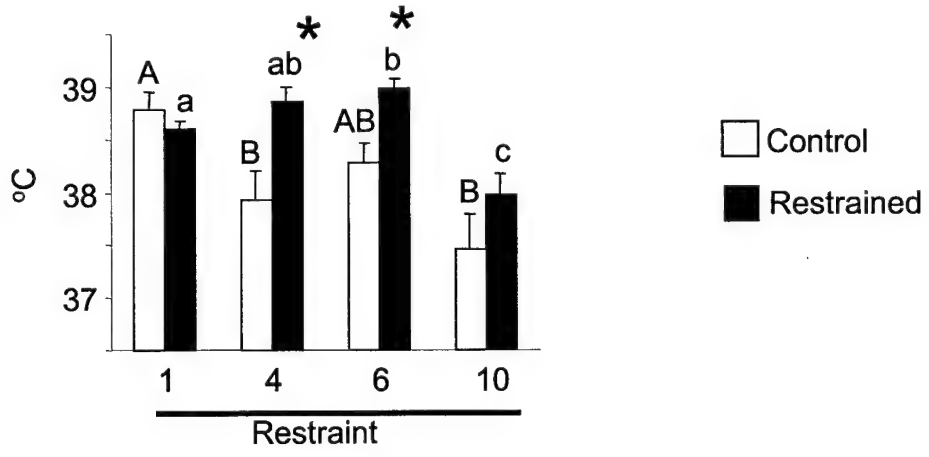


Figure 2.

A: Corticosterone in Rats Restrained on 10 Consecutive Days



B: Rectal Temperatures of Rats Restrained on 10 Consecutive Days



C: Leptin in Rats Restrained on 10 Consecutive Days

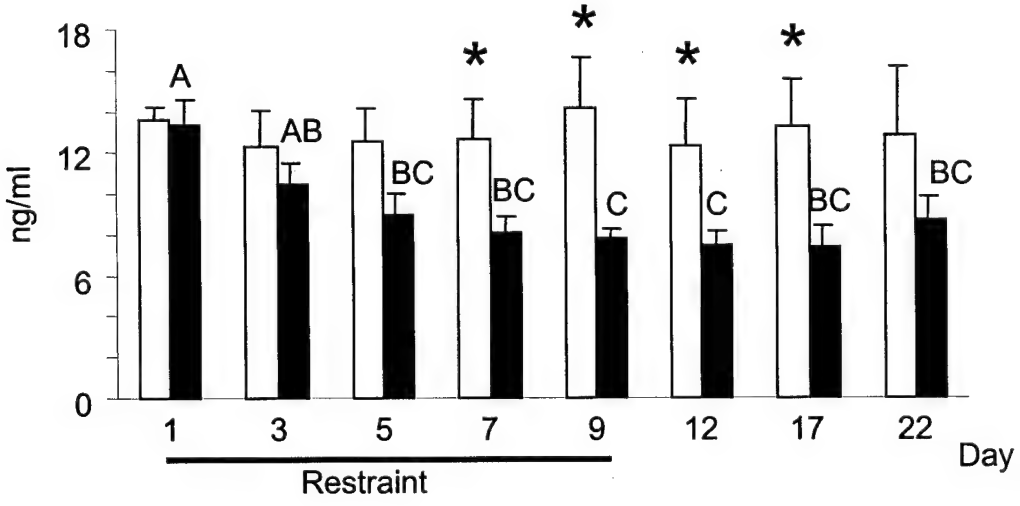
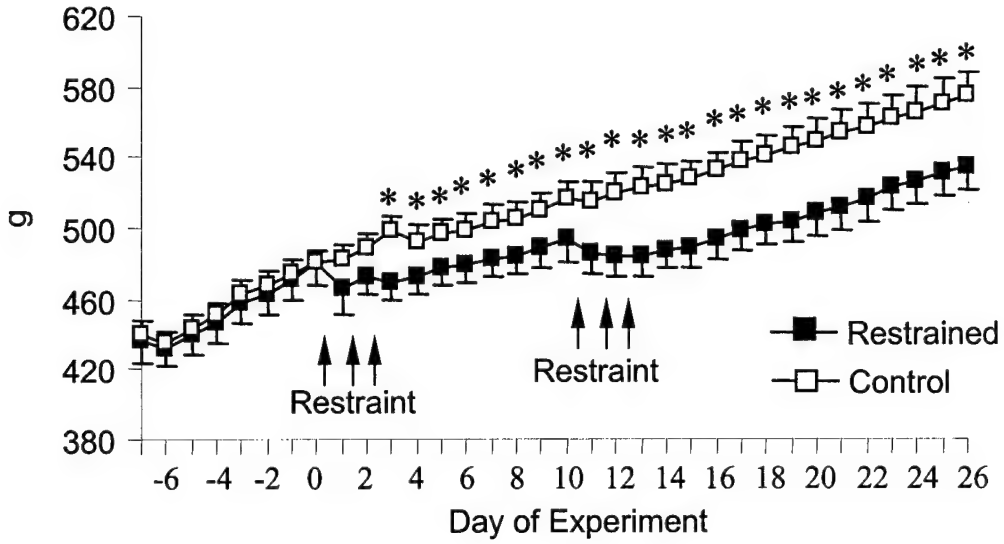
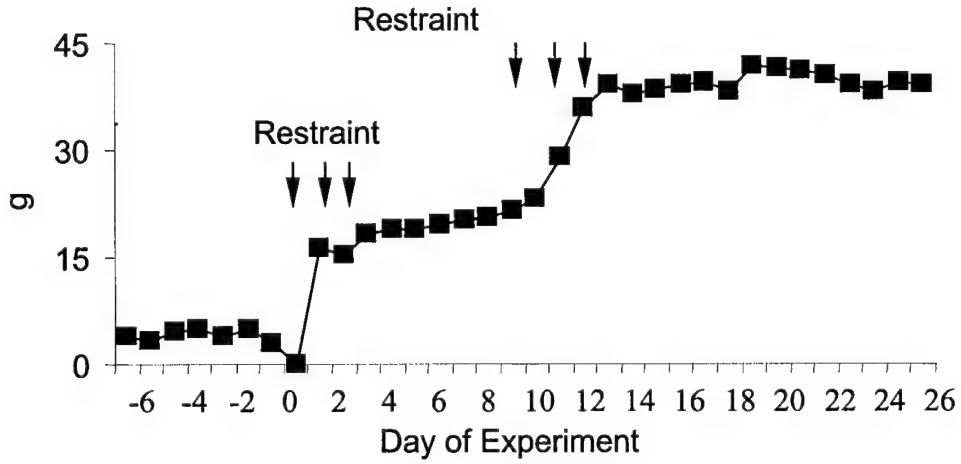


Figure 3.

A: Daily Body Weights of Rats Exposed to Two Bouts of Repeated Restraint



B: Difference in Body Average Weights of Control and Restrained Rats



C: Daily Food Intake of Rats Exposed to Two Bouts of Repeated Restraint

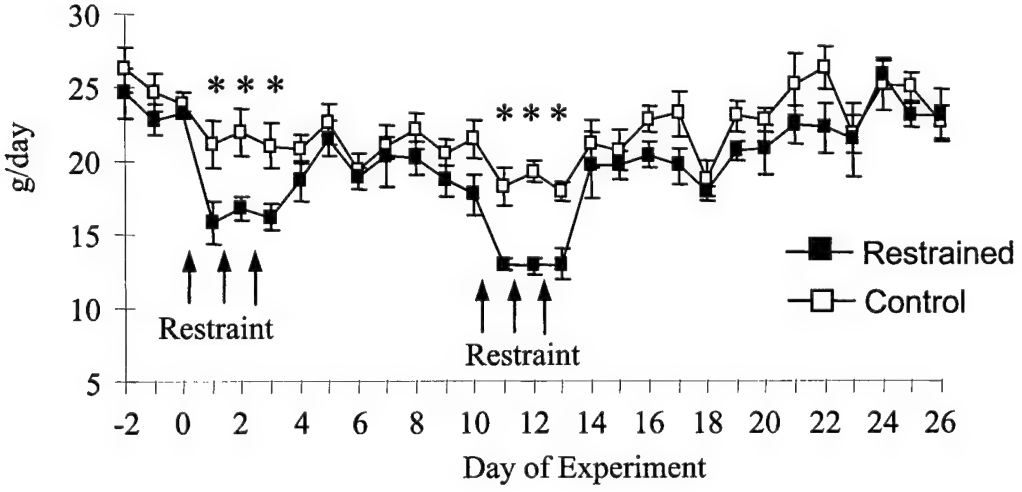
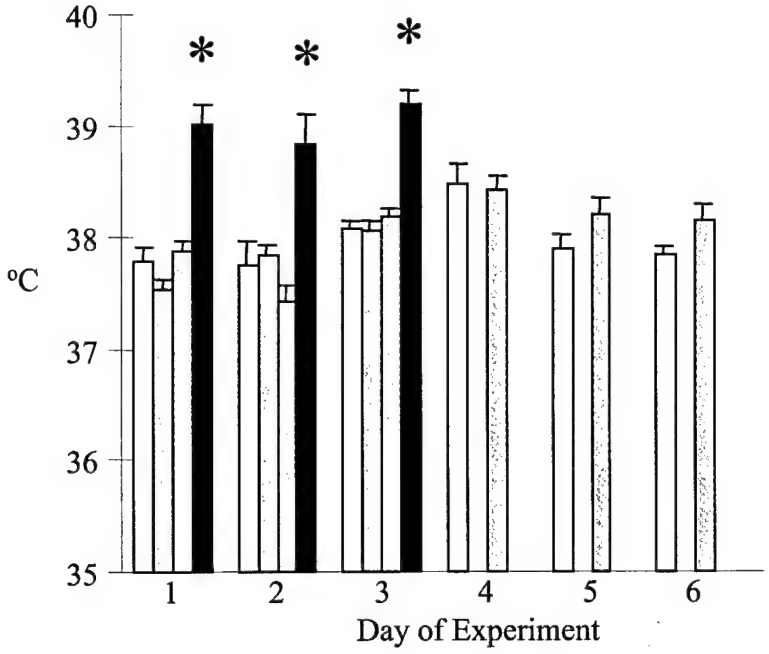
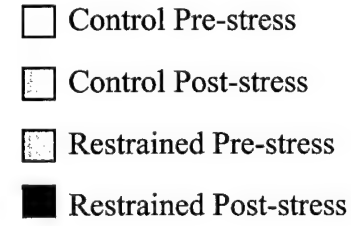


Figure 4.

A: Rectal Temperature During First Bout of Restraint





B: Rectal Temperature During Second Restraint

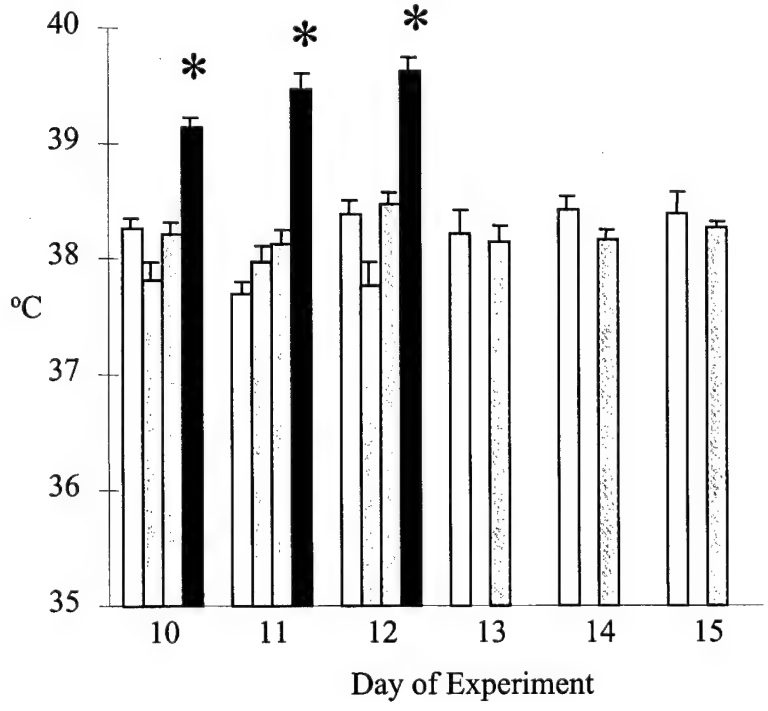
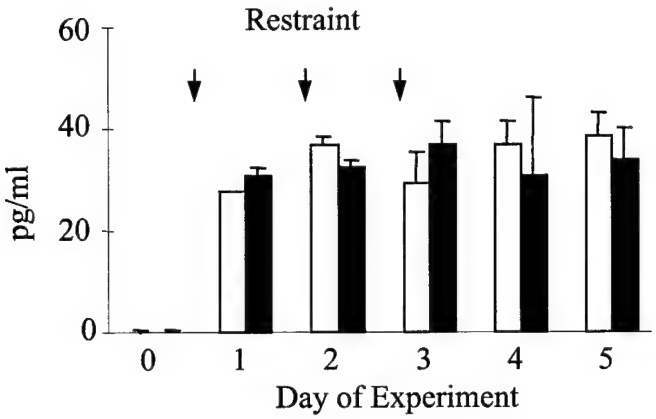
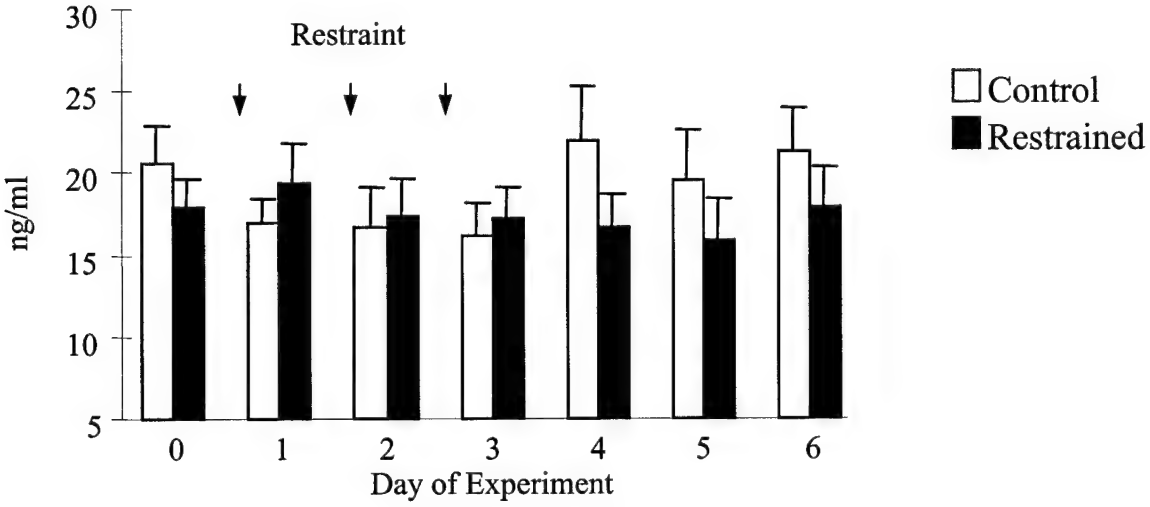


Figure 5.

A: Free IL-6 Leptin During the First Bout of Repeated Restraint



B: Leptin During the First Bout of Repeated Restraint



C: Leptin During the Second Bout of Repeated Restraint

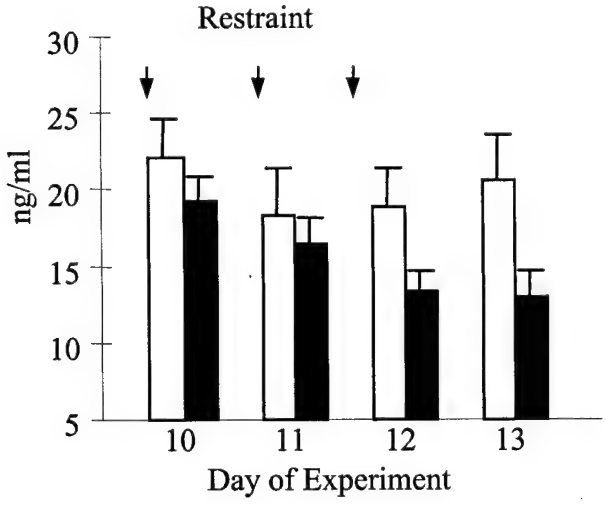
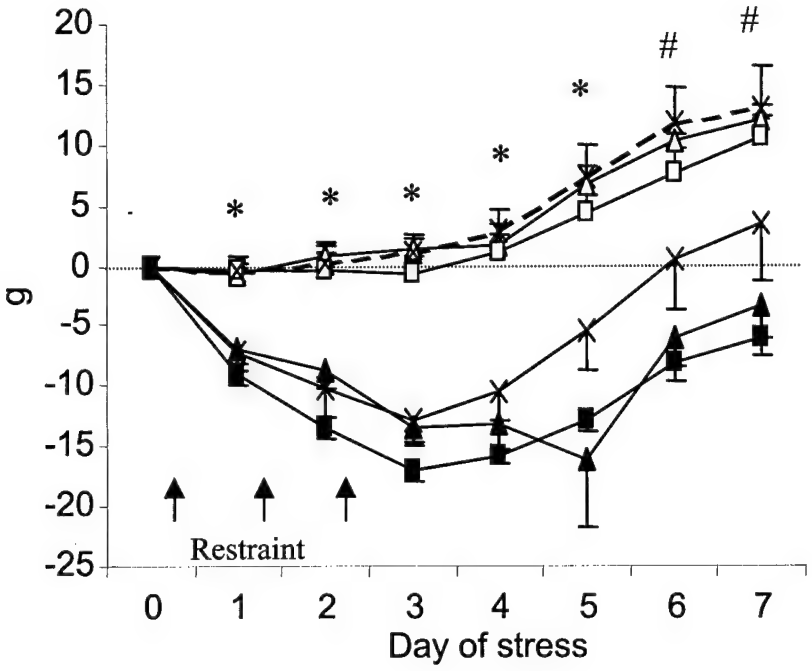
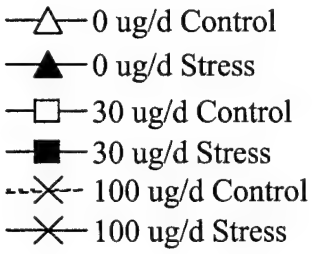


Figure 6.

A: Post-Stress Change in Body Weight of Leptin Infused Rats





B: Post-Stress Food Intake Of Leptin Infused Rats

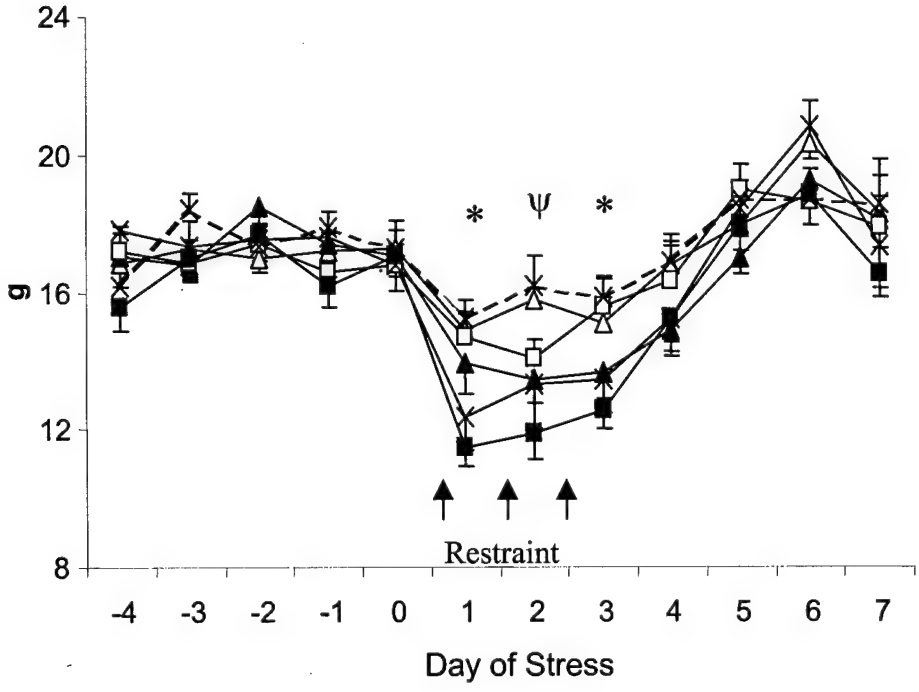
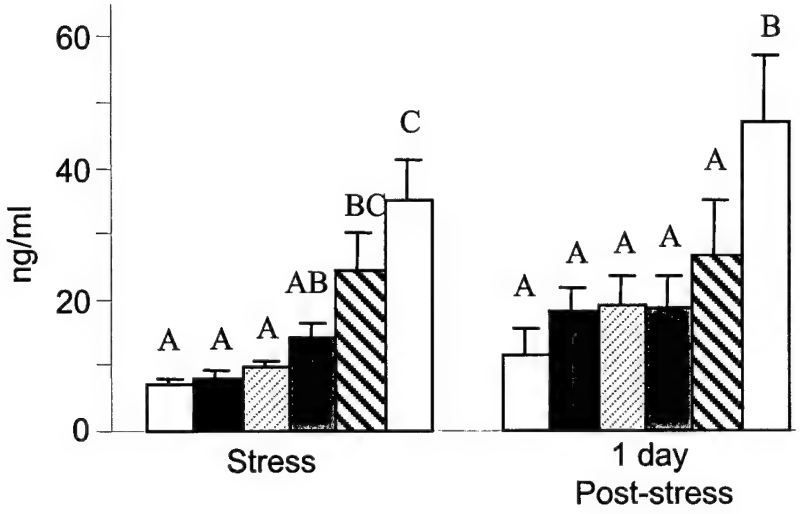


Figure 7.

A: Leptin in Restrained Leptin Infused Rats



B: Corticosterone in Restrained Leptin Infused Rats

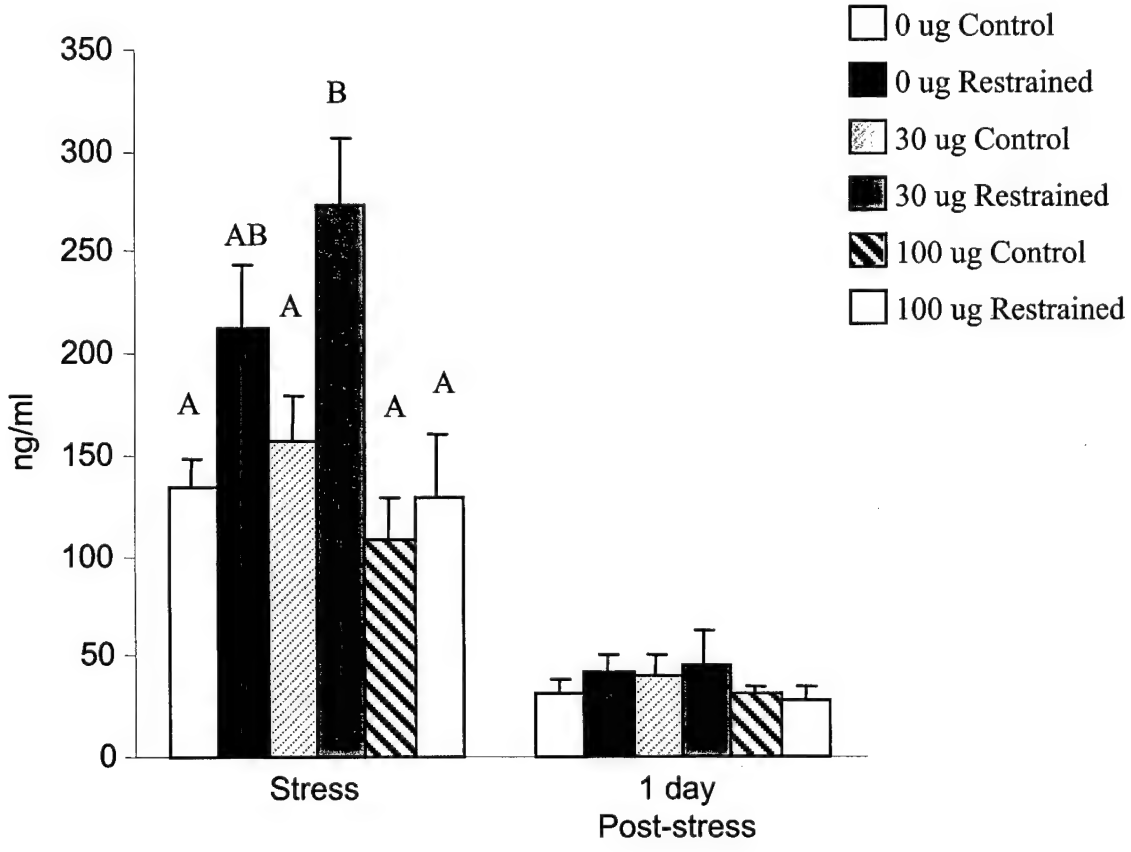
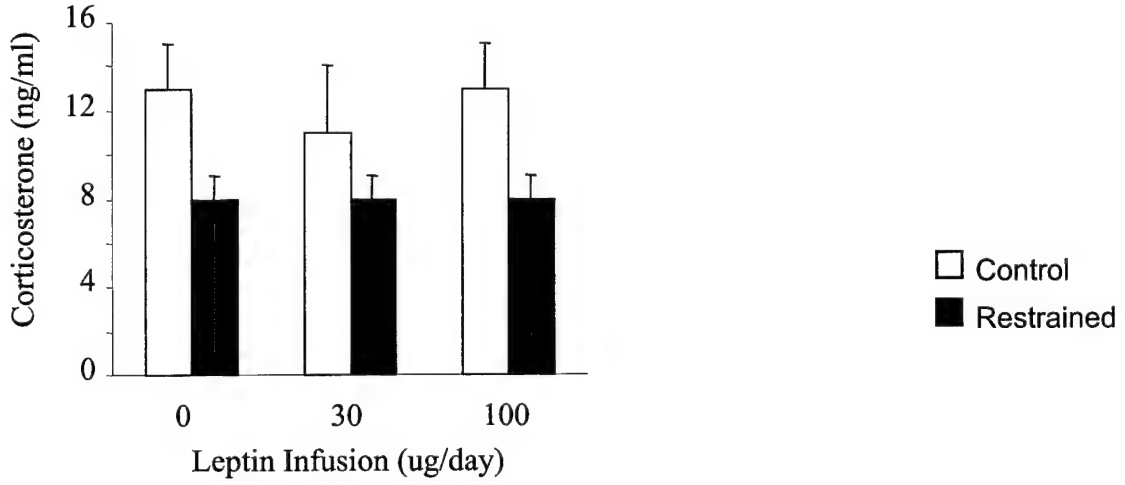


Figure 8.

A: Corticosterone at the end of Leptin Infusion



B: Leptin at the end of Leptin Infusion

C: Leptin mRNA at the end of Leptin Infusion

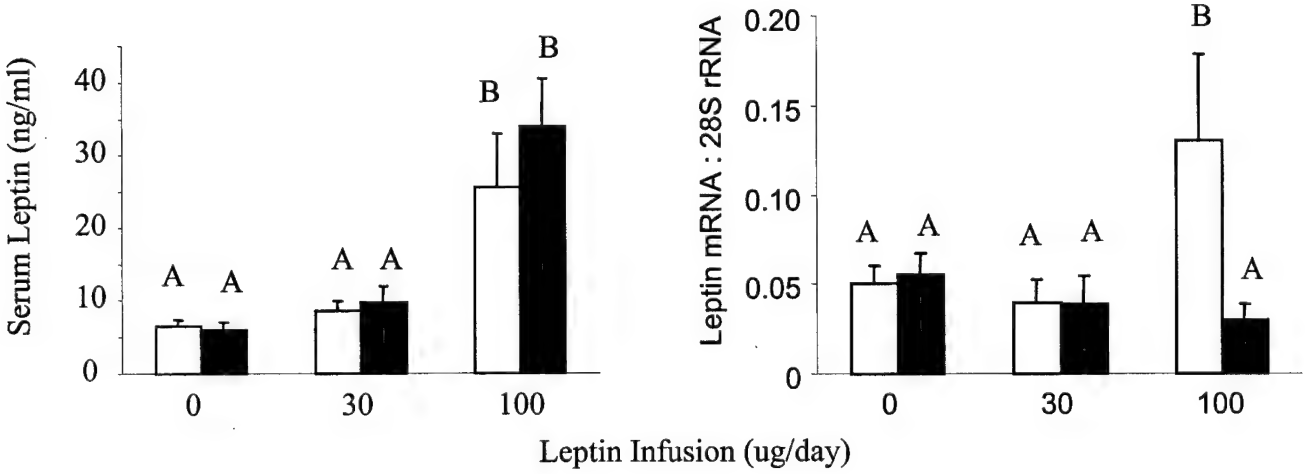
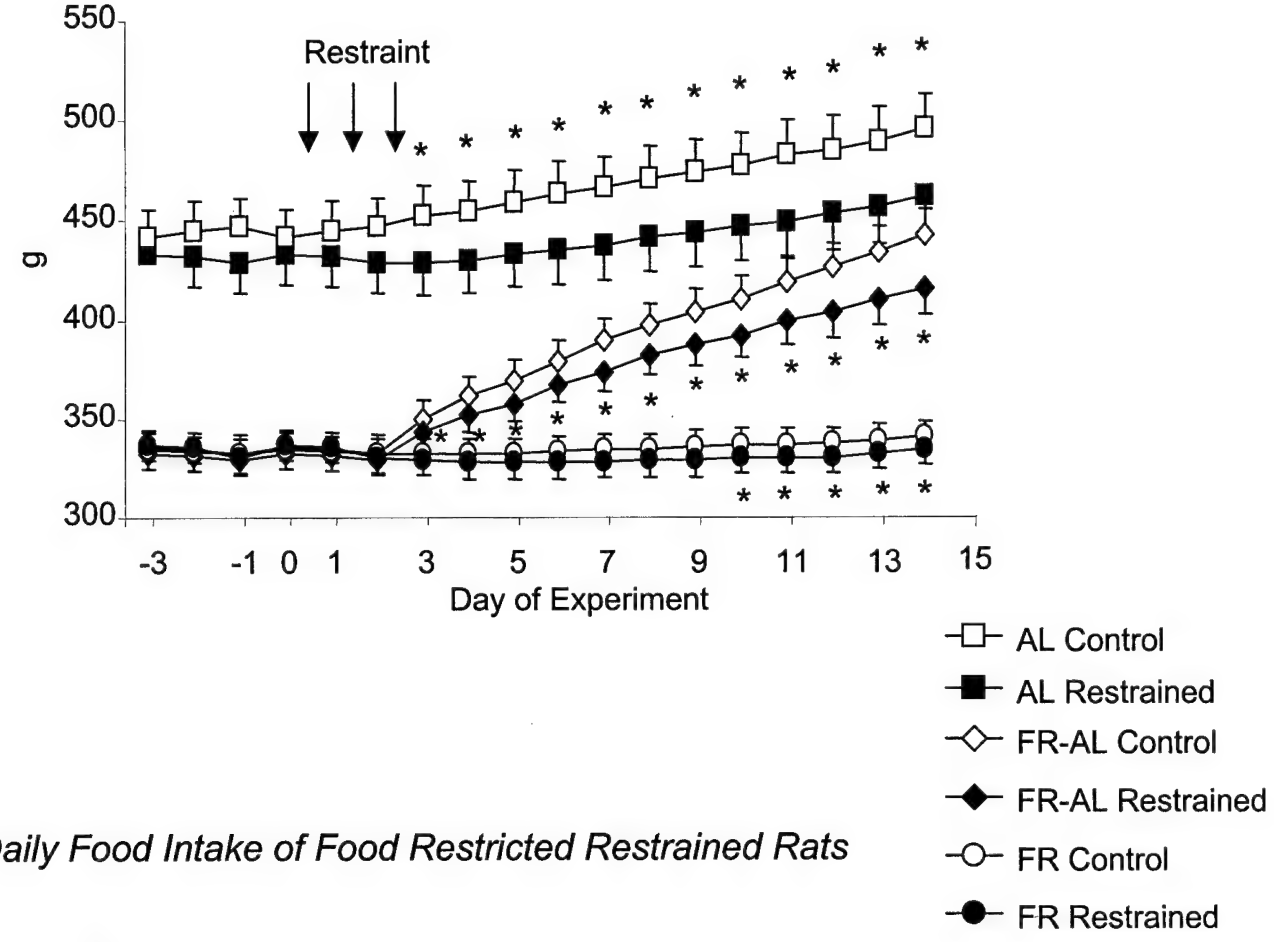


Figure 9.

A: Daily Body Weight of Food Restricted Restrained Rats



B: Daily Food Intake of Food Restricted Restrained Rats

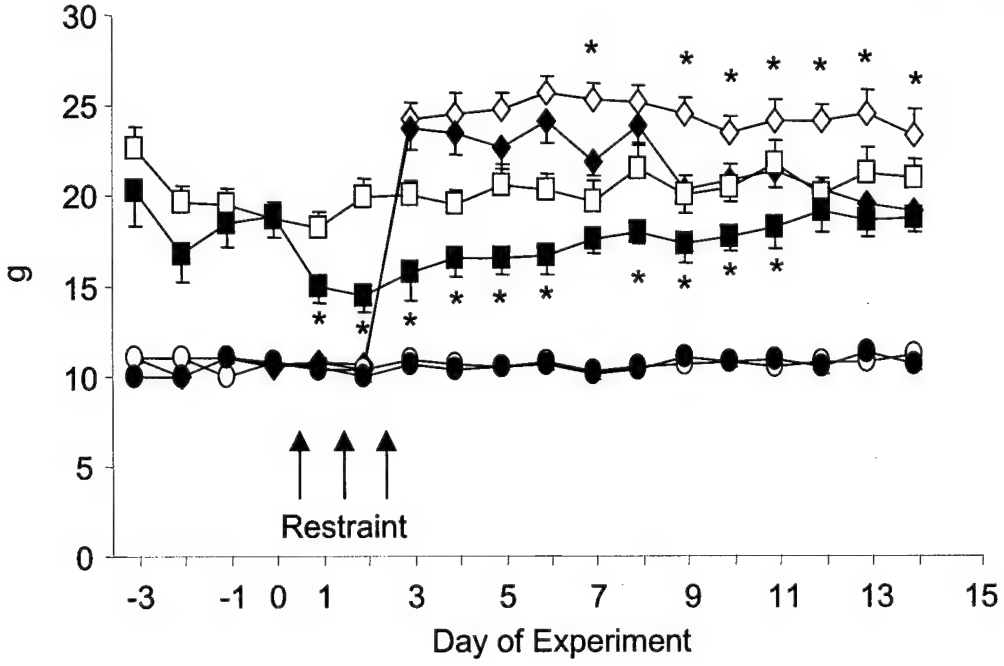
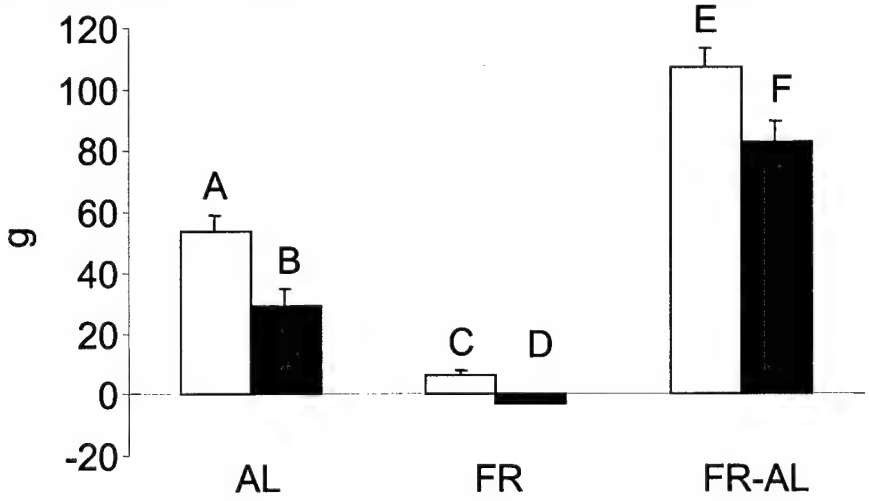
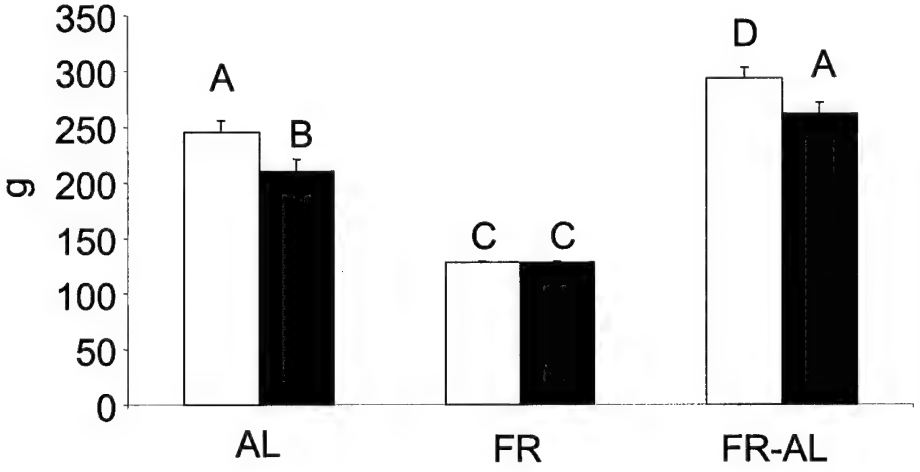


Figure 10.

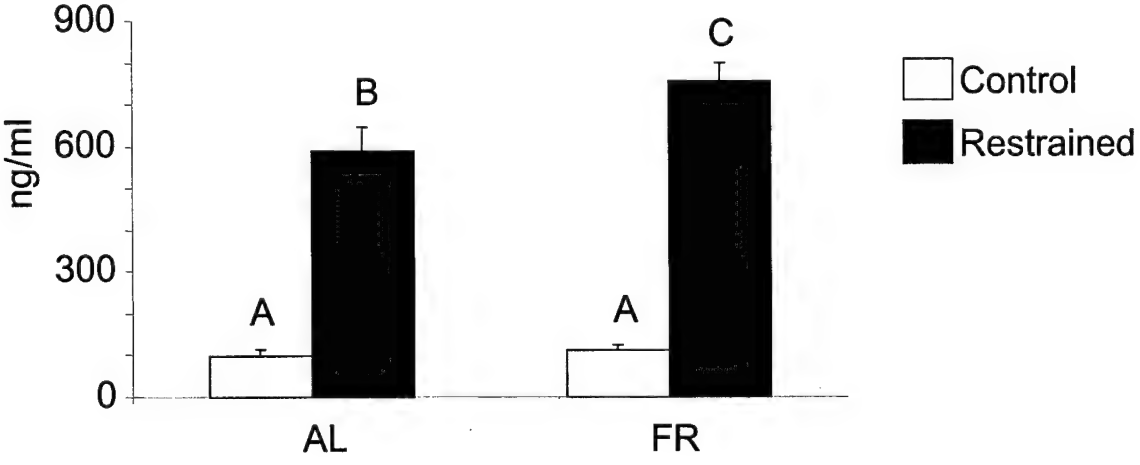
A: Weight Change in Restrained Food Restricted Rats



B: Cumulative Food Intake of Restrained Food Restricted Rats



C: 1 Hour Corticosterone in Restrained Food Restricted Rats



Over-Expression of Agouti Protein and Stress Responsiveness in Mice

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ABSTRACT

HARRIS, R.B.S., J. ZHOU. M. SHI, S. REDMANN, Jr., R.L. MYNATT, D..H. RYAN,. Over-expression of agouti protein and stress responsiveness in mice. PHYSIOL BEHAV XX(X) 000-000, 2001. Ectopic over-expression of agouti protein, an endogenous antagonist of melanocortin receptors, linked to the β-actin promoter (BAPa) in mice produces a phenotype of yellow coat color, Type II diabetes, obesity and increased somatic growth. Spontaneous over-expression of agouti increases stress-induced weight loss. In these experiments, other aspects of stress-responsiveness were tested in 12-week-old male wild-type mice and BAPa mice. Two hours of restraint on three consecutive days produced greater increases in corticosterone and post-stress weight loss in BAPa than wild-type mice. In Experiment 2, anxiety-type behavior was measured immediately after 12 minutes of restraint. This mild stress did not produce many changes indicative of anxiety but BAPa mice spent more time in the dark side of a light-dark box and less time in the open arms of an elevated plus maze than restrained wild type mice. In a defensive withdrawal test, grooming was increased by restraint in all mice but the duration of each event was substantially shorter in BAPa mice, possibly due to direct antagonism of the MC4-R by agouti protein. Thus, BAPa mice showed exaggerated endocrine and energetic responses to restraint stress with small differences in anxiety-type behavior compared with wild-type mice. These results are consistent with observations in other transgenic mice in which the melanocortin system is disrupted, but contrast with reports that acute blockade of central melanocortin receptors inhibits stress-induced hypophagia. Thus, the increased stress-responsiveness in BAPa mice may be a developmental compensation for chronic inhibition of melanocortin receptors.

Key words: Melanocortin receptors, αMSH, ACTH, restraint stress, anxiety, body weight.

INTRODUCTION

Agouti protein is an endogenous antagonist of melanocortin receptors (MC-R) which is usually only expressed in the hair follicle, regulating coat color (3). A protein with a similar sequence, agouti related protein (AgRP) is expressed in brain (23) and adrenal cortex (1) and a human homologue of AgRP (23), which also was identified as agouti signaling protein (ASP) (33), is expressed in adipose tissue, skin, testes, ovaries, liver, kidney and heart (33). The endogenous ligands for melanocortin receptors are derived from proopiomelanocortin (POMC). Alpha-melanocyte stimulating hormone (αMSH) is an endogenous agonist of all melanocortin receptors except the ACTH receptor (MC2-R) (25) and ACTH is a ligand for all receptor sub-types.

Ectopic over-expression of agouti, due either to autosomal dominant expression (34) or by linking the agouti gene to the β-actin promoter (17), results in a phenotype of yellow coat color, Type II diabetes, adult onset obesity, increased incidence of tumors and increased somatic growth. The complexity of the phenotype is due to agouti antagonizing the five MC-R sub-types (numbered 1 through 5). The affinity of agouti protein varies across the sub-types of receptor, showing a high affinity for MC1-R (18), responsible for the modulation of coat color, and for MC4-R but a lower affinity for MC3-R (15). Both MC3-R and MC4-R are expressed in the brain, have been implicated in the regulation of body weight (10) and account for the obesity in agouti mice. The affinity of agouti protein for MC2-R has not been determined but it has been demonstrated that agouti will displace ACTH from MSH receptors (18). AgRP is a more specific antagonist of melanocortin receptors, having a very high affinity for MC4-R, a lower affinity for MC3-R and no effect on MC1-R or MC2-R, the ACTH receptor (23).

Stress initiates a complex cascade of responses that include endocrine, neurological and behavioral events. Many of these responses are initiated by the central release of corticotrophin releasing factor (CRF) and its related protein, urocortin (6). Evidence for a relationship between stress and agouti protein is equivocal. Studies with transgenic animals indicate that inhibition of the melanocortin system may increase sensitivity to stress. It has been reported that A(y) mice, a spontaneous mutation that results in ectopic agouti expression, lose more weight in response to stress that do their wild-type controls (5), and mice that are MC4-R deficient show an exaggerated hypophagic response to CRF infusion (19). In contrast, acute central administration of a specific MCR4 antagonist partially prevents stress-induced weight loss and hypophagia in Lister-Hooded rats (32) and central infusion of AgRP does not have any effect on CRF-induced hypophagia in Wistar rats (8), suggesting that the CRF and melanocortin systems are independent.

The effects of agouti protein on stress-induced responses other than hypophagia and weight loss have not been elucidated. Because it is not known whether agouti protein blocks the MC2-R, it is not clear whether mice over-expressing agouti would have an enhanced or diminished endocrine response to stress. Blocking the MC2-R receptor might inhibit stress-induced glucocorticoid release, however, CRF can stimulate the adrenal gland by activating the sympathetic nervous system (9). Alternatively, inhibition of the negative feedback of ACTH on CRF release via the MC3-R receptor (26). and blockade of the well-established inhibitory effect of αMSH on the release of pro-inflammatory cytokines (13) might enhance the response to stress.

In order to obtain a better understanding of the effects of agouti protein on different aspects of the stress response we tested the effect of restraint stress on food intake, body weight,

corticosterone release, brain monoamine concentrations and anxiety-type behaviors in transgenic mice over-expressing agouti protein linked to the β-actin promoter (BAPa) (17). The BAPa transgenic mice were developed to demonstrate that the phenotype of lethal yellow (A^y) and viable yellow (A^{vy}) mice were attributable to over-expression of agouti protein and were not caused by the additional structural changes near the agouti locus in the mice with spontaneous mutations (17). In the original study several lines of BAPa mice were developed to ensure that there was no significant transgene effect on the mouse phenotype. The animals used in these experiments were derived from line BAPa20, which were shown to have the highest level of agouti expression and to develop more severe obesity and diabetes than the mice with lower levels of agouti expression (17).

In the first study we tested the effects of repeated acute restraint stress on food intake, body weight and corticosterone release of the mice because we have previously demonstrated that repeated restraint stress causes a chronic down-regulation of body weight in rats (12). In Experiment 2, we tested the effect of a brief exposure to restraint on anxiety-type behavior in three different assays. At the end of this study hypothalamic CRF concentrations and frontal cortex and brain stem monoamines were measured because it is established that dopamine and norepinephrine release in the frontal cortex and brain stem are increased by stress (4) and associated with anxiety-type behavior (21). The results of the two experiments demonstrate that over-expression of agouti protein results in an exaggeration of only some of the behavioral and endocrine responses to restraint stress.

MATERIALS AND METHODS

Experiment 1: The Effects of Repeated Restraint Stress on Body Weight, Food Intake and Endocrine Response

Twenty wild-type and 20 heterozygote transgenic (BAPa) 12-week old male mice were housed individually on grids inside shoebox cages in a room maintained at 74 $\pm$ 2°F on a 12 hour light:dark cycle with lights on at 6:00 a.m. A piece of plastic pipe (1.5" internal diameter) was placed in each cage to minimize the stress of being housed on grids. All mice had free access to chow (Purina mouse Chow 5015, Ralston Purina, St. Louis, MO) and water. Body weights and food intakes, corrected for spillage, were measured daily. Mice were obtained from a breeding colony maintained at the Pennington Center and all experimental procedures were approved by the Pennington Biomedical Research Center Institutional Animal Use and Care Committee.

At the end of one week of baseline measurements, the mice in each genotype were divided into two weight-matched groups (wild-type, 30.5 $\pm$ 0.6 g: BAPa, 37.8 $\pm$ 1.1 g) and one group from each genotype was restrained for two hours (7:00 - 9:00 a.m.) on each of three consecutive days (Days 0, 1 and 2). The restrained mice were placed in Perspex restraining tubes (Plas Labs, Lansing, MI) and control mice were moved to the same room and were deprived of food and water for the period of restraint. Thirty minutes after the start of restraint on the first and last days of restraint (Days 0 and 2) and at an equivalent time following 30 minutes of food deprivation on the day after the end of restraint (Day 3), a small blood sample (~100 ul) was collected from each mouse by tail-bleeding, for measurement of serum corticosterone (Corticosterone RIA; ICN Pharmaceuticals, Costa Mesa, CA).

Nine days after the end of repeated restraint, mice that had been exposed to repeated restraint were placed in restraint tubes for 30 minutes before decapitation and controls were housed in the same room without access to food or water for 30 minutes before decapitation. The mice were killed between 8.00 and 11.00 a.m. with animals from each group sacrificed across time. Blood was collected for measurement of serum corticosterone, insulin (Rat Insulin

RIA kit; Linco Research Inc.), free tumor necrosis factor alpha (TNFα: Mouse TNFα Quantikine kit; R&D Systems, Minneapolis, MN) and leptin (Mouse Leptin RIA kit: Linco Research, St. Louis, MO). Epididymal and mesenteric white fat, intrascapular brown fat (IBAT) and the thymus gland were weighed.

Experiment 2: The Effect of Acute Restraint on Anxiety-Type Behavior and Central Monoamine Concentrations.

Twenty heterozygote BAPa and 28 wild type 12-week old male mice were housed individually in shoebox cages and body weight was recorded daily. After five days, the mice in each genotype were divided into two weight-matched groups and one group was restrained for 12 minutes. Immediately after restraint, the stressed and control mice were tested for anxiety behavior in the defensive withdrawal test, described below. The short restraint was selected to induce a level of anxiety that was mild enough to enable detection of differences in response between the two genotypes. Six days later, stressed mice were restrained again for 12 minutes and anxiety-type behavior of control and restrained mice was measured in a light-dark box, as described below. Six days after this test, a third measure of anxiety behavior was made in an elevated plus maze. Ten days after the last anxiety test, the restrained mice were decapitated immediately after 30 minutes restraint stress. Controls were housed in the same room as restrained mice, without food or water, for 30 minutes before decapitation. Trunk blood was collected for measurement of plasma corticosterone (Corticosterone RIA: ICN Pharmaceuticals, Costa Mesa, CA)). The thymus and adrenal glands were weighed and the hypothalamus (14) was snap frozen for measurement of corticotrophin releasing factor (CRF) content, as described below. Frontal cortex and brain stem were dissected (14) and snap frozen for determination of catecholamine content, described below.

Defensive Withdrawal Test:

The defensive withdrawal behavioral apparatus consisted of a brightly illuminated 45-cm square open field with a white floor and 30 cm high black wall. A black cylindrical chamber (length 10 cm, diameter 6.5 cm), open at one end, was secured to the floor lengthwise next to one wall, 20 cm from the corner. The mouse was placed in the chamber and an Ethovision Image Motion Tracking System (Version 1.70, Noldus Information Technology, Wageningen, Netherlands) recorded activity during a 5-minute session. The following behaviors were measured: latency to leave the chamber, distance moved, velocity, number of entries into the center zone, time spent in the center zone, number and duration of rears, number and duration of grooming events, number of exits from the chamber and time spent out of the chamber. In this test an increase in anxiety would be measured as increased time spent in the dark chamber, fewer entries into the center zone, less rearing behavior and more grooming.

Light-Dark Box Test:

The light-dark box consisted of two polyvinylchloride boxes (23.5 x 23.5 x 23.5 cm) connected by a semicircular hole (9cm high and 5cm wide) through which the mouse could move. One box had black walls, floor and ceiling and the other had white walls and floor and was illuminated by a 75-watt bulb placed 3 inches above the box. The mouse was placed in the dark box and the following behaviors were recorded during a 5-minute session: latency to escape the dark box, number of exits from the dark box, total time spent in the light box, mean time spent in the light box. In this test increased anxiety would be indicated by a preference for the dark box.

Elevated Plus Maze Test:

The elevated plus maze consisted of four 10 cm wide and 48 cm long arms set at 90° to one another, extending from a central platform (10 x10 cm). Two opposing arms were open and the other two arms were closed with 30-cm high black walls. The apparatus was elevated 50 cm above the floor. At the start of the test the mouse was placed on the center platform facing an open arm. The following behaviors were recorded during a 5-minute test period: number of entries into the closed arms, time spent in the closed arms, number of entries into the open arms, time spent in the open arms. In this test anxiety would be indicated by a preference for the closed arms of the maze.

Hypothalamic CRF Concentration.

The hypothalamus from each mouse was sonicated in ice-cold 1% trifluoroacetic acid and centrifuged at 10,000 xg for 20 minutes at 4°C. CRF was extracted using C-18 Sep-columns according to manufacturers instructions (Peninsula Laboratories, Inc. San Carlos, CA). The final eluate was dried in a Speedvac centrifuge and dissolved in assay buffer. Aliquots of the extract were used to determine CRF content using an EIA kit (Peninsula Laboratories, Inc.). The protein concentration of each sample was determined by BCA protein assay kit (BCA; PIERCE, Rockford, IL) and results were expressed as ng CRF/mg of protein.

Brain Monoamine Measurements.

Frontal cortex and brain stem were each sonicated in 300 ul 0.1 M perchloric acid, 0.1 M EDTA. The homogenate was centrifuged at 14,000 xg, 4°C for 10 minutes. The supernatant was centrifuged in the same conditions and then separated by HPLC (ESA INC., Chelmsford, MA) as described previously (36), to measure the concentrations (pg/ug protein) of norepinephrine (NE), its metabolite 3-methoxy,4-hydroxyphenylethyleneglycol (MHPG), serotonin (5-HT) and its

metabolite 5-hydroxyindoleacetic acid (5-HIAA) and dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). The ratios of NE/MHPG, 5-HT/5-HIAA and of DA/DOPAC were used as indices of monoamine metabolism.

Statistical Analysis.

Body weight and food intake data were analyzed by repeated measurements of variance. Two way ANOVA with post-hoc Duncan's multiple range test was used for each day's serum corticosterone level, hypothalamus CRF protein content, hormone measurements and brain monoamines. For the anxiety behavior tests, the data was analyzed by two way ANOVA with Tukey's multiple comparisons test, except for those measures that were considered to be categorical data, in which case a chi-square test was used with pair-wise comparisons adjusted by multiple comparisons. The SAS system version 6.12 was used for computations. Data are presented as means ± sem.

RESULTS

Experiment 1

Repeated restraint stress caused weight loss in both wild-type and BAPa mice, with a greater weight change over time in the BAPa mice (Figure 1: Stress: $P<0.02$, Gene: $P<0.05$, Day:$P<0.001$, Day x Stress: $P<0.01$). Control mice, exposed to the mild stress of being moved to a novel environment during the period of restraint, also lost weight with a greater response in BAPa than wild-type mice. By the end of the experiment, wild-type controls had returned to their pre-stress weights and wild-type restrained animals were gaining weight, but had not returned to their pre-stress weight. In contrast, there was no indication that either group of BAPa mice were recovering the weight that they had lost on the days of restraint. Stress inhibited food intake of both wild-type and BAPa mice on the three days of restraint (Figure 2: Stress: $P<0.05$,

Gene: NS, Day:$P<0.001$, Day x Gene: $P<0.07$). The hypophagia was reversed as soon as stress ended but there was no evidence of over-eating during the post-stress period (Figure 2). The food intakes of control wild-type mice did not change during the experimental period, but there was a decline in the intake of the BAPa control mice during the stress period that was reversed within four days of the end of stress.

Restraint stress significantly increased serum corticosterone concentrations measured 30 minutes after the start of restraint on the first and last day of repeated restraint and again after an acute exposure to restraint at the end of the experiment (Figure 3: Stress: $P<0.01$, Gene: NS, Int: $P<0.005$). There were no differences in corticosterone of the two control groups but corticosterone was higher in stressed BAPa mice than in stressed wild-type mice on all three days (Figure 3). There was a gradual increase in circulating corticosterone concentrations in both wild-type and BAPa mice with repeated exposure to restraint. The day after the end of repeated restraint serum corticosterone concentrations were significantly higher ($P<0.02$) in BAPa than wild-type mice, but there was no difference between control and restrained mice within the genotypes (Figure 3). At the end of the experiment there was no significant effect of restraint on fat pad weights or serum leptin, insulin or free-TNFα concentrations, as shown in Table 1. The BAPa mice were significantly fatter ($P<0.001$) than the wild-type mice, they had larger IBAT depots and they also had significantly higher serum concentrations of insulin and leptin ($P<0.001$). There were no differences in serum free-TNFα concentrations.

Experiment 2.

Stress caused a significant change in all of the anxiety-type behaviors measured in the defensive withdrawal apparatus immediately after 12 minutes of restraint. Restraint significantly increased latency to exit the chamber, reduced the number of exits from the chamber, distance

traveled in the open field, velocity, the number of rears and the total duration of rearing in both BAPa and wild-type mice (Table 2: $P<0.05$). The number of grooming events and the total time spent grooming was significantly increased in restrained mice compared with controls (Table 2: $P<0.02$). The BAPa mice showed the same number of grooming events as the wild-type mice, but the total time spent grooming was decreased by almost 60% in BAPa compared with wild-type mice (Table 2: $P<0.02$). The BAPa mice showed a significant increase in the number of rears, compared with wild-type mice, but the total time spent rearing was the same for the two genotypes.

Behavioral measures made in the light-dark box indicated that restrained BAPa mice were more anxious than the restrained wild-type mice (Table 3). That is, restraint increased the latency to escape from the dark box (Table 3: $P<0.05$) but the difference was significant only in BAPa mice. Stress reduced the number of exits from the dark box for both wild-type and BAPa mice but the number of exits was significantly lower for BAPa than wild-type mice in both control and stressed conditions ($P<0.05$). There was no effect of genotype or restraint on the total amount of time spent in either the light or the dark box.

The third test for anxiety-type behaviors in the elevated plus maze also indicated an increase in anxiety in BAPa mice, compared with wild-type mice (Table 4). Specifically, restrained wild-type mice spent less time in the closed arms of the maze than their controls and increased the number of entries into the open arms and the total amount of time spent in the open arms of the maze (Table 4: $P<0.05$). In contrast, there was no effect of restraint on the amount of time spent by BAPa mice in the open or closed arms of the maze, but the number of entries into the closed arms was significantly decreased following restraint (Table 4: $P<0.05$), indicating that the stressed BAPa mice increased the duration of each visit to the closed arms.

Neither restraint stress nor genotype had any effect on hypothalamic CRF content, measured immediately after 30 minutes of restraint (Figure 4). There were small differences in plasma ACTH with a significant interaction ($P<0.05$) between stress and genotype, such that ACTH was increased in restrained wild type mice, but not BAPa mice, compared with their respective controls (Figure 4: $P<0.05$). Restraint stress significantly increased plasma corticosterone concentrations in both BAPa and wild-type mice (Figure 4: $P<0.01$), but the increase was greater in BAPa mice (Figure 4: Gene x Stress: $P<0.07$), confirming the results from Experiment 1.

Frontal cortex norepinephrine metabolism, expressed as MHPG/NE, was significantly increased by 30 minutes of restraint in BAPa compared with wild type mice (Figure 5: $P<0.05$) although there were no significant differences in the concentrations of either NE or MHPG. There were no significant differences in the concentrations of serotonin, dopamine, their metabolites or the ratio of metabolite to neurotransmitter in the frontal cortex (data not shown). DOPAC concentration was significantly increased in the brain stem of restrained animals (Figure 5: $P<0.05$). The difference was significant for wild-type but not BAPa mice, in which DOPAC content in tissue from non-restrained controls was already at the level found in stressed mice (Figure 5). The ratio of DOPAC:DA was increased by restraint but did not reach statistical significance (Figure 5: $P<0.07$). There were no differences in the concentrations of 5-HT, NE or their metabolites in the brain stem (data not shown). The BAPa mice were heavier than the wild-type mice, but the 12 minute exposures to restraint had no effect on body weight of either genotype (data not shown). There were no effects of either genotype or stress on the weights of adrenal or thymus glands of the mice (data not shown).

DICUSSION

The overall results from the present experiments indicate that ectopic over-expression of agouti protein increases certain aspects of stress responsiveness in mice. Specifically, there was a greater post-stress effect on body weight, a greater restraint-induced stimulation of corticosterone and increased stress-induced norepinephrine turnover in the frontal cortex of BAPa mice, compared with wild-type mice subjected to the same stress. The results of behavioral measures of anxiety-type behavior did not demonstrate a clear difference in response between the two genotypes, possibly because the 12 minute restraint was insufficient to induce a significant level of anxiety in any of the mice. The increased stress responsiveness, as indicated by sustained weight loss, in BAPa mice is similar to that of A(y) mice (5), that also over-express agouti protein. The A(y) mice show an exaggerated hypophagic response to stress even though stress-induced corticosterone release is the same as in wild-type mice.

The increase in serum corticosterone concentrations of restrained BAPa mice, compared with restrained wild-type mice, may be explained in several ways. First, agouti related protein has a low affinity for MC2-R and it is possible that during development, chronic, partial antagonism of MC2-R leads to an increased level of expression of the ACTH receptor in the adrenal cortex, which might cause the animals to be more responsive to stress-induced ACTH release. Alternatively, if the blockade of ACTH activity by agouti protein is fully effective, then there may be a compensatory adjustment in the sensitivity of the adrenal gland to direct stimulation by the sympathetic nervous system. As mentioned above, A(y) mice show an exaggerated hypophagic response to stress even though stress-induced corticosterone release is the same as in wild-type mice. Because the animals in both studies were 8 week old males (5), the difference in corticosterone response to restraint stress between BAPa and A(y) mice seems

to be attributable to either the level of agouti expression or the background strain of the mice, which was C57BL/6J for the A(y) mice and FVB for the BAPa mice. There are several instances in which the phenotype associated with a single genetic mutation is modulated by the genetic background of the mouse (20).

Although weight loss in the post-stress period was greater for restrained BAPa than wild-type animals, the increased level of corticosterone during stress cannot account for the maintained weight loss because non-stressed BAPa mice lost weight, even though their serum corticosterone concentrations were not different from those of non-stressed wild type animals. In addition, we have previously shown that antagonism of central CRF receptors, by infusion of αhCRF into the third ventricle, prevents stress-induced weight loss but does not prevent stress-induced corticosterone release (28). Although restraint caused a greater release of corticosterone in the BAPa than the wild-type mice, ACTH concentrations were lower in restrained BAPa than wild-type mice after 30 minutes of restraint. There was no significant effect of stress on ACTH in either genotype of mice, possibly because of the long time interval between the initiation of stress and sample collection. The blunted ACTH response in restrained BAPa mice, compared with wild-types, would be consistent with them being either more sensitive to ACTH, or having adapted to reliance upon neural activation of the adrenal gland.

The weight loss of restrained mice was associated with a decreased food intake during the stress period. The direct effect of restraint on food intake was reversed within 24 hours of the end of restraint in both wild-type and BAPa mice, but both control and restrained BAPa mice ate less for several days following the end of restraint than they had been eating during the control period. The maintenance of a lower body weight during the post-stress period, despite food intake returning to control levels, suggests that energy expenditure of the mice was

increased by repeated restraint stress. Although the BAPa and wild-type mice had relatively similar levels of food intake in non-stress conditions, the BAPa mice were heavier, which implies that they are normally more efficient than wild-type mice. Observations that A^y mice have a lower core temperature than wild-type controls (16) is consistent with overepxression of agouti protein resulting in reduced rates of thermogenesis. The weight loss in control BAPa mice that experienced the mild stressors of being moved to an experimental room during the period of restraint and the handling associated with tail-bleeding is additional evidence of increased stress-responsiveness in these animals. Again, this result is consistent with the report that food intake of A(y) mice was inhibited, compared with wild-type mice, when the animals were moved from group housing to individual caging and during the days following a 1 hour restraint stress (5). It has been reported that MC4-R deficient mice lose more weight and eat less than wild-type mice in response to central CRF administration, whereas there is no difference in the response of the two genotypes to central urocortin injection (19). These observations imply that the interaction between stress and the melanocortin system in transgenic mice is extremely complex. Both CRF and urocortin activate all sub-types of the CRF receptor (29), therefore, the exaggeration of the response to CRF in MC4-R deficient mice cannot be attributed to an effect at either the receptor or post-receptor level.

In contrast to the increased responsiveness to CRF in MC4-R knockout mice (19), acute blockade of central MC3-R and MC4-R receptors with AgRP does not change CRF-induced hypophagia in Wistar rats (8). In addition, selective antagonism of the MC4-R receptor partially prevents hypophagia and weight loss in immobilized rats (32) and does not influence CRF-induced hypophagia (31). It is not unusual for there to be disparities between expectations based on pharmacological studies and phenotypes that develop in transgenic mice (27). Further studies

are needed to clarify the basis for the contrasting results from BAPa mice, which have developed with a modified melanocortin system, and rats in which specific melanocortin receptors are acutely blocked. One possible explanation is that rats and mice are different, another is that melanocortin peptides act as a negative feedback signal, probably via MC3-R, to inhibit CRF release in the brain (26). Over-expression of agouti protein would block the negative feedback and allow an exaggerated response to stress-induced CRF release. In contrast, when exogenous CRF is injected into the brain, AgRP may block the binding of ACTH to MC3-R, but it will not change the amount of CRF present. In Experiment 2, there was no difference in hypothalamic CRF concentration of BAPa and wild type mice after 30 minutes of restraint stress, but it is possible that a site specific increase in CRF concentration was masked by analyzing the whole hypothalamic area, or that the time point of measurement was not appropriate.

An alternative explanation for the extended weight loss response to stress in BAPa mice may be the blockade of melanocortin receptors at a site other than the hypothalamus. For example, agonism and antagonism of melanocortin receptors in the brainstem has potent inhibitory or stimulatory effects on feeding and weight change (11). Finally, because the acute studies with rats indicate that CRF-induced hypophagia is independent of the melanocortin system, it is possible that the increased stress-responsiveness in BAPa mice is mediated by another system that is up-regulated in transgenic mice in order to compensate for the chronic blockade of melanocortin receptors. One possibility is increased tone of the catecholaminergic system. In Experiment 2 we found an increase in brain norepinephrine turnover in the frontal cortex of BAPa mice, compared with wild-type mice, and circulating concentrations of norepinephrine and dopamine are increased in POMC deficient mice (35). The circulating catecholamines have to be of peripheral neural origin as POMC-deficient mice have no

functional adrenal gland and very low circulating concentrations of epinephrine (35), which is almost exclusively produced by the adrenal medulla.

It is well established that αMSH can inhibit the immune response to endotoxic stress (22) by a mechanism that includes the inhibition of release of pro-inflammatory cytokines such as TNFα and interleukin 1 (IL-1)(24). Stress promotes a pro-inflammatory response (37) and it is possible that the increased responsiveness of BAPa mice to restraint stress was secondary to an uncontrolled release of cytokines that inhibited food intake, caused fever and wasting of body tissue. We found no differences in the serum concentrations of free-TNFα in any of the groups of mice following 30 minutes of restraint, but this does not exclude the possibility of an exaggerated immune response as the sampling time may not have been optimal for detection of a difference between genotypes and other cytokines, such as IL-1, that inhibit food intake, promote corticosterone release and cause anxiety-type behaviors (2).

In Experiment 2 we found few indications of increased anxiety-type behavior in restrained BAPa mice, compared with wild-type mice. The animals were tested in three different assays, all of which were based on exploration of a novel environment that incorporated an aversive, brightly illuminated area. Inclusion of the defensive withdrawal test, which involves an open-field component, allowed separation of anxiety-related behavior from genotype differences in spontaneous activity measured in non-stressed mice. The tests were carried out in the same order for all animals, therefore, it is possible that repeated exposure to restraint stress modified anxiety behavior in some of the tests and may explain why there was little indication of anxiety in either wild-type or BAPa mice in the Elevated Plus Maze, which was thelast of the three tests to be conducted. Our primary interest, however, was to compare BAPa and wild-type mice that had been exposed to the same procedures. There were no genotype differences in

locomotor activity in the defensive withdrawal test, but BAPa mice showed a significantly greater number of rears of short duration, so that the total time spent rearing was the same for both genotypes. As rearing may be indicative of exploration of the environment, this would suggest either that the BAPa mice were not different from wild-type mice because they both exhibited the same number of rears, or that BAPa mice were more anxious because the duration of each rearing event was shorter. The BAPa mice also showed a change in grooming behavior compared with wild-type mice. BAPa mice did not change the number of grooming events recorded in the defensive withdrawal test, but the total time spent grooming was reduced by almost 60% in both control and restrained BAPa mice, compared with wild-type animals. Decreased grooming can indicate decreased anxiety, but in these mice it may have been specifically associated with the over-expression of agouti protein because acute blockade of MC4-R in rats prevents αMSH-induced grooming but does not change locomotion or exploration in non-stressed rats (30). The results also imply that stress-induced grooming is largely mediated by the melanocortin system (7). Although there were no substantial differences in behavior of the mice under the test conditions used here, 30 minutes of restraint caused a greater increase in norepinephrine turnover in the frontal cortex and basal dopamine concentrations in the brain stem were increased in BAPa mice, compared with wild-type animals. Because dopamine and norepinephrine release in the frontal cortex and brain stem are associated with anxiety-type behavior (21), it is possible that we would have detected differences in behavior of the BAPa and wild-type mice in response to a more severe stressor.

In summary, the results of the present study suggest that ectopic over-expression of agouti protein increases the stress-responsiveness of mice, measured by sustained changes in energy balance, and by restraint-induced corticosterone release and norepinephrine turnover in

the frontal cortex. These results are similar to those reported for A(y) mice (5), that also over-express agouti protein, and the observation that MC4-R deficient mice have an increased sensitivity to the inhibitory effects of CRF on food intake (19). It is not clear, however, whether the increased responsiveness in transgenic mice is simply due to antagonism of melanocortin receptors because acute inhibition of central melanocortin receptors in rats does not prevent CRF-induced hypophagia (31) and inhibits stress-induced weight loss (32). Thus, it is possible that the increased stress-responsiveness of transgenic mice in which the melanocortin system has been partially, or totally, inactivated is due to the up-regulation of another system to compensate for chronic inhibition of melanocortin activity.

FOOTNOTE

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FIGURE LEGENDS

Figure 1: Weight change from the day before stress of BAPa and wild-type mice exposed to 2 hours of restraint stress on each of 3 days. Values are means $\pm$ sem for groups of 10 mice. An asterisk indicates a significant difference ($P<0.05$) between stressed and control mice within each genotype.

Figure 2: Daily food intakes for BAPa and wild-type mice exposed to 2 hours of restraint stress on each of three days. Values are means + sem for groups of 10 mice. Asterisks indicate significant differences ($P<0.05$) between control and restrained mice.

Figure 3: Serum corticosterone measured at different times during Experiment 1. Data are means + sem for groups of 10 mice. Values that do not share a common superscript on any specific day are significantly different at $P<0.05$. Acute stress measurements were made on trunk blood collected from mice that were killed immediately following 30 minutes of restraint, 9 days after the end of repeated restraint stress.

Figure 4: Hypothalamic CRF protein concentration, plasma ACTH and plasma corticosterone concentrations measured at the end of Experiment 2 immediately after 30 minutes of restraint. Data are means + sem for groups of 10 BAPa or 14 wild-type mice. Values for ACTH or corticosterone that do not share a common superscript are significantly different at $P<0.05$.

Figure 5: Frontal cortex norepinephrine and brain stem dopamine concentrations measured at the end of Experiment 2 immediately after 30 minutes of restraint. Data are means + sem for groups of 10 BAPa or 14 wild-type mice.

Table 1: Organ weights and serum hormones for wild type and BAP mice exposed to restraint stress.

	Wild-type mice		**BAPa mice**	
	Control	*Restrained*	*Control*	*Restrained*
Organ Weights (mg)				
Epididymal fat	769 ± 107^{A}	781 ± 130^{A}	1217 ± 124^{B}	1353 ± 80^{B}
Mesenteric fat	290 ± 46^{A}	316 ± 54^{A}	592 ± 104^{B}	527 ± 51^{B}
IBAT	149 ± 16^{A}	137 ± 15^{A}	181 ± 18^{B}	192 ± 17^{B}
Thymus	37 ± 3	35 ± 3	41 ± 4	37 ± 3
Serum Hormones				
Insulin (ng/ml)	0.70 ± 0.13^{A}	0.49 ± 0.06^{A}	2.45 ± 0.62^{B}	2.66 ± 0.71^{B}
Leptin (ng/ml)	10.4 ± 1.5^{A}	7.3 ± 0.9^{A}	31.5 ± 6.8^{B}	30.7 ± 4.3^{B}
Free TNFα (pg/ml)	115 ± 21	90 ± 13	106 ± 19	76 ± 18

Data are means ± sem for groups of 10 mice killed 9 days after the end of repeated restraint. The restrained mice were exposed to 30 minutes of restraint immediately before decapitation and controls were housed in the same room without food or water for the 30 minute period. Values for specific fat pads that do not share a common superscript are significantly different at $P<0.05$.

Table 2. Results from the Defensive Withdrawal test in Experiment 2.

	Wild-type mice		**BAPa mice**	
	Control	*Restraint*	*Control*	*Restraint*
Latency to leave chamber (sec)	12 ± 4^{A}	48 ± 9^{B}	16 ± 6^{A}	56 ± 12^{B}
Entries into the Center Zone	11 ± 2	9 ± 2	11 ± 1	7 ± 3
Time in the Center Zone (sec)	18 ± 9	20 ± 5	16 ± 2	10 ± 4
Distance moved (cm)	2172 ± 206^{A}	1674 ± 242^{B}	2007 ± 241^{A}	1546 ± 276^{B}
Velocity (cm/s)	7.4 ± 0.7^{A}	5.7 ± 0.8^{B}	6.9 ± 0.8^{AB}	5.4 ± 0.9^{B}
Number of rears	23 ± 2^{A}	12 ± 3^{B}	48 ± 6^{C}	22 ± 5^{A}
Time spent rearing (sec)	48 ± 6^{A}	22 ± 5^{B}	50 ± 9^{A}	25 ± 7^{B}
Number of grooming events	2 ± 0^{A}	5 ± 2^{B}	1 ± 0^{A}	4 ± 1^{B}
Time spent grooming (sec)	6 ± 1^{A}	30 ± 9^{B}	2 ± 1^{C}	12 ± 4^{A}
Number of exits from chamber	5 ± 1^{A}	3 ± 1^{B}	6 ± 1^{A}	3 ± 1^{B}
Time spent out of chamber (sec)	258 ± 12^{A}	207 ± 26^{B}	254 ± 13^{A}	186 ± 35^{B}

Data are means $\pm$ sem for groups of 10 (BAPa) or 14 (wild-type) mice. Behaviors were measured during a 5 minute session that started immediately after 12 minutes of restraint in restrained mice or an equivalent time in the experimental room without food or water for the control mice. Two-way analysis of variance did not show any interaction between genotype and stress on behavior. Values for a specific behavior that do not share a common superscript are significantly different at $P<0.05$.

Table 3. Results from the Light-Dark box test in Experiment 2.

	Wild type mice		**BAPa mice**	
	Control	Restraint	Control	Restraint
Latency to escape dark box (sec.)	43 ± 6^{A}	56 ± 10^{AB}	32 ± 4^{A}	68 ± 13^{B}
Number of exits from dark box	8.4 ± 0.6^{A}	7.5 ± 0.6^{A}	7.4 ± 0.6^{B}	4.2 ± 1.0^{C}
Total time in dark box (sec)	212 ± 9	197 ± 11	203 ± 8	210 ± 26
Time spent in light box (sec.)	88 ± 9	103 ± 12	96 ± 6	90 ± 26
Mean time spent in light box (sec.)	10 ± 1	15 ± 2	14 ± 2	21 ± 7

Data are means $\pm$ sem for groups of 10 (BAPa) or 14 (wild-type) mice. Behaviors were measured during a 5 minute session that started immediately after 12 minutes of restraint in restrained mice or an equivalent time in the experimental room without food or water for the control mice. Two-way analysis of variance did not show any effect of genotype or any interaction between genotype and stress on behavior. Values for a specific behavior that do not share a common superscript are significantly different at $P<0.05$.

Table 4. Results from the Elevated Plus Maze test in Experiment 2.

	Wild-type mice		**BAPa mice**	
	Control	*Restraint*	*Control*	*Restraint*
Number of entries into closed arms	13 ± 1^{AB}	12 ± 1^{AB}	14 ± 1^{A}	10 ± 1^{B}
Time spent in closed arms (sec.)	140 ± 14^{A}	90 ± 7^{B}	136 ± 14^{A}	133 ± 19^{A}
Number of entries into open arms	10 ± 1^{A}	14 ± 1^{B}	12 ± 2^{AB}	10 ± 1^{A}
Time spent in open arms (sec.)	94 ± 15^{A}	154 ± 10^{B}	93 ± 16^{A}	98 ± 10^{A}

Data are means $\pm$ sem for groups of 10 (BAPa) or 14 (wild-type) mice. Behaviors were measured during a 5 minute session that started immediately after 12 minutes of restraint in restrained mice, or an equivalent time in the experimental room without food or water for the control mice. Two-way analysis of variance did not show any effect of genotype or any interaction between genotype and stress on behavior. Values for a specific behavior that do not share a common superscript are significantly different at $P<0.05$.

Figure 1

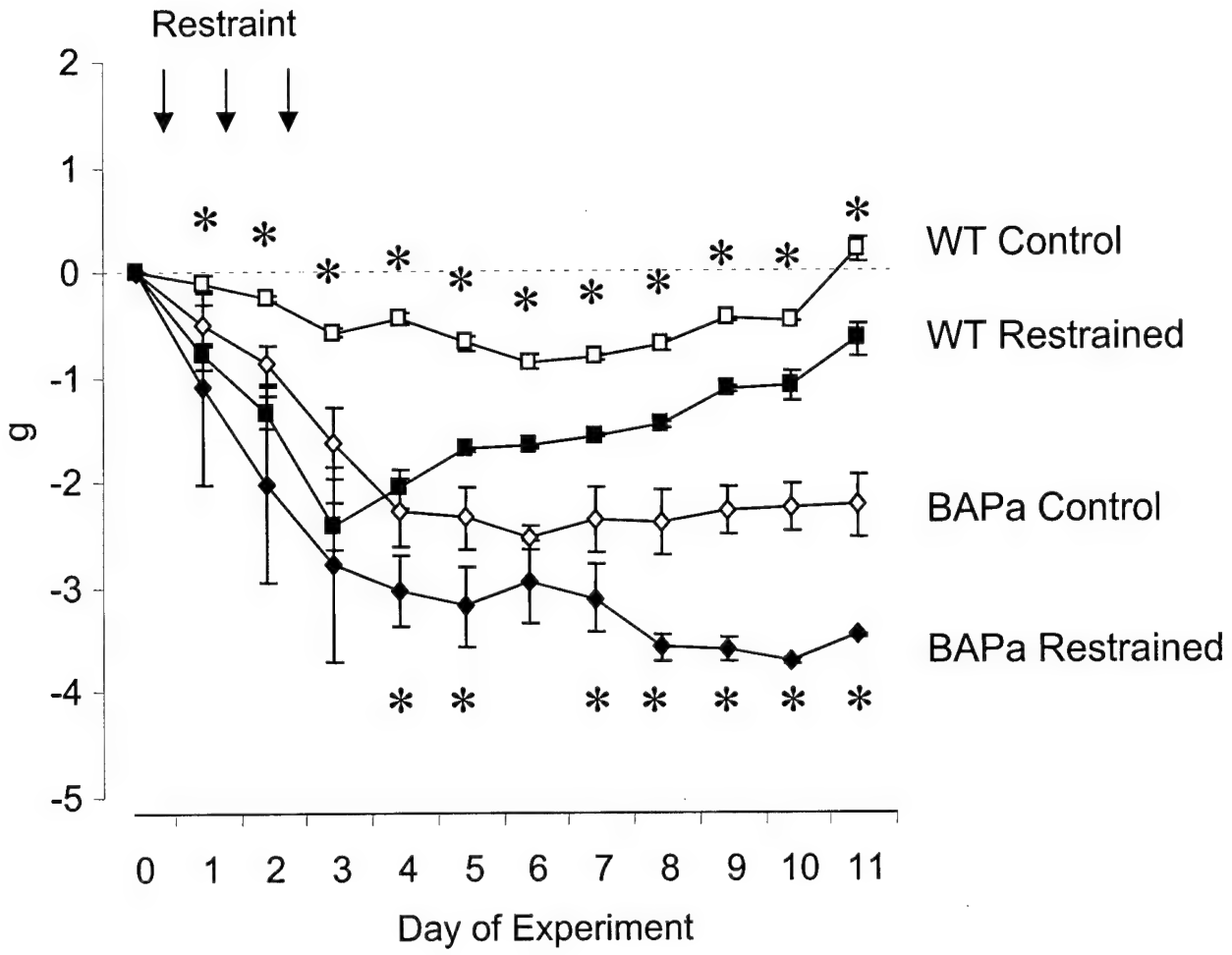
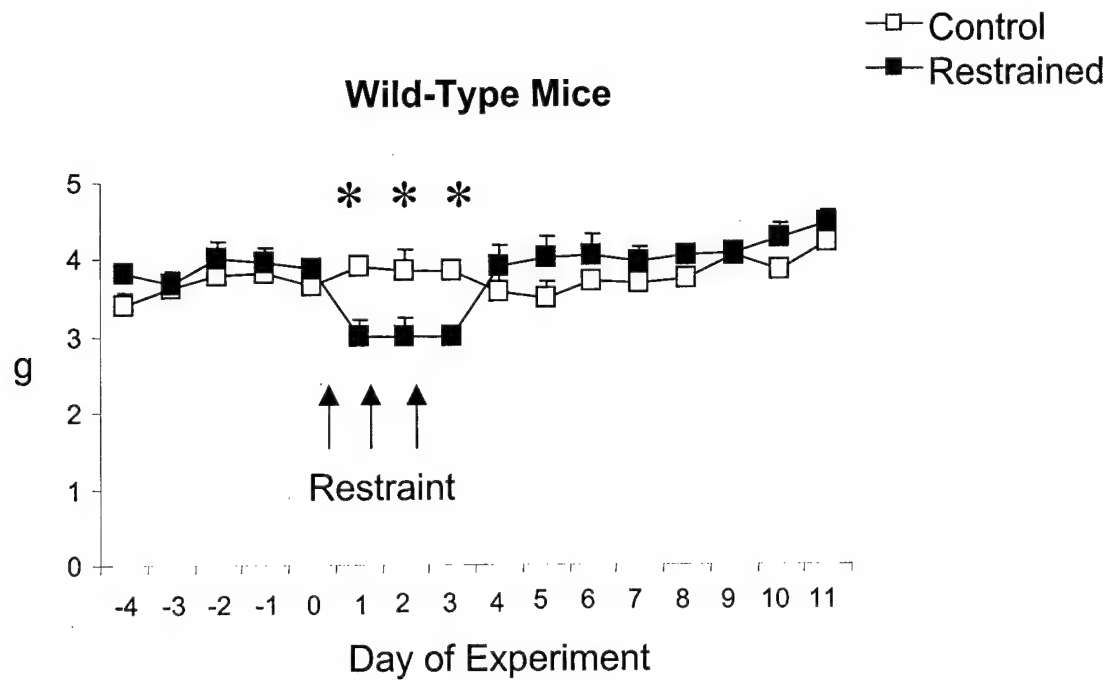
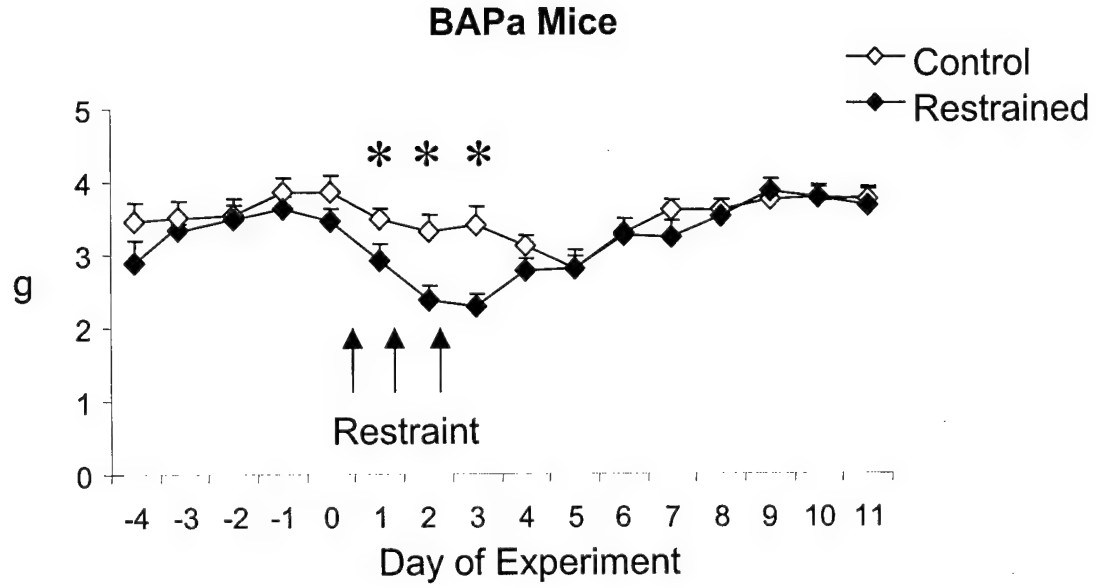

Restraint
2
1
0
-1
-2
-3
-4
-5
g
0 1 2 3 4 5 6 7 8 9 10 11
Day of Experiment
WT Control
WT Restrained
BAPa Control
BAPa Restrained

Figure 2


Control
Restrained
Wild-Type Mice
* * *
g
5
4
3
2
1
0
Restraint
-4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11
Day of Experiment


BAPa Mice
Control
Restrained
* * *
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4
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Restraint
-4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11
Day of Experiment

Figure 3

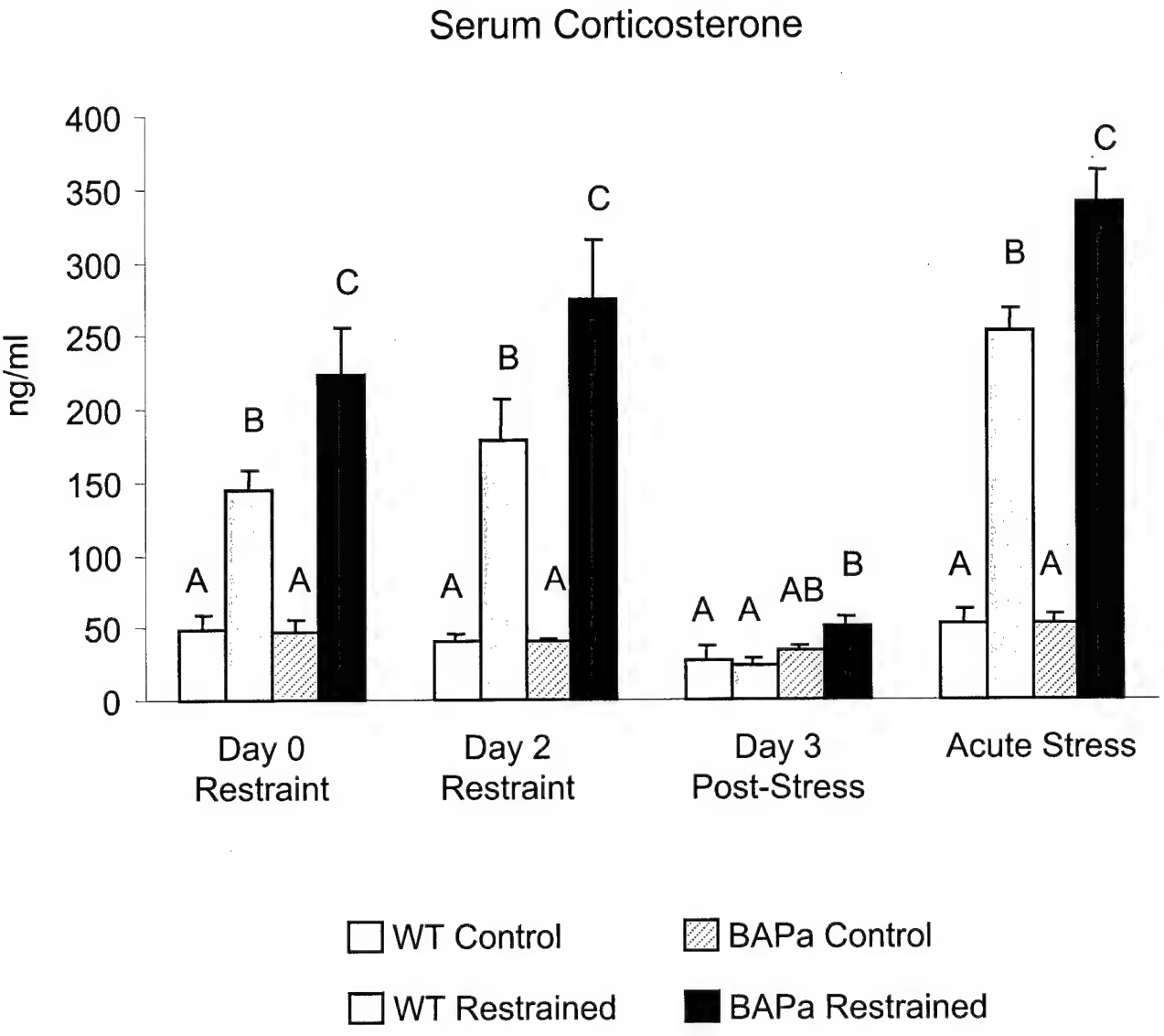
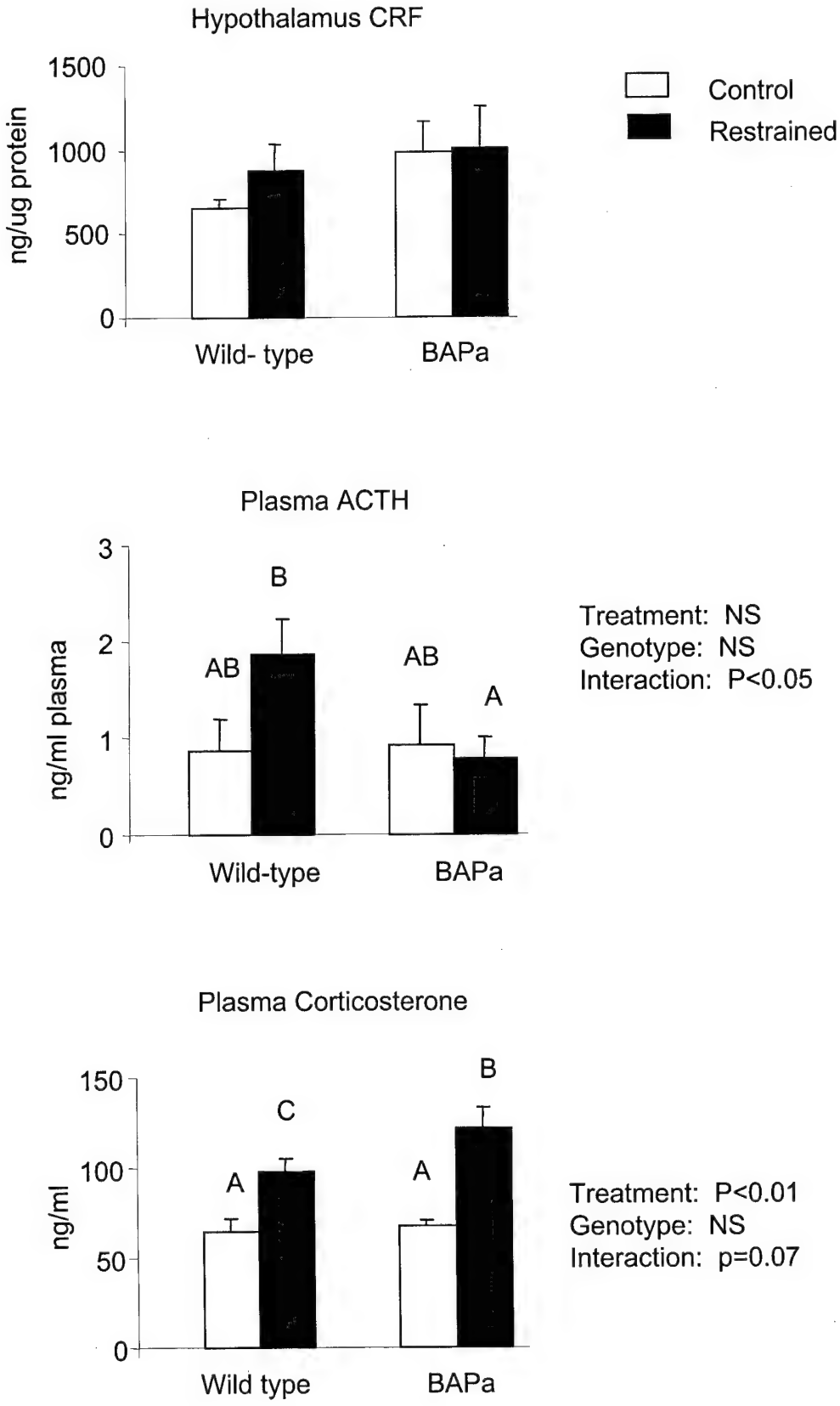

Serum Corticosterone
ng/ml
400
350
300
250
200
150
100
50
0
A
B
C
A
B
C
A
A
AB
B
A
B
C
Day 0
Restraint
Day 2
Restraint
Day 3
Post-Stress
Acute Stress
WT Control
BAPa Control
WT Restrained
BAPa Restrained

Figure 4


Hypothalamus CRF
1500
1000
500
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ng/ug protein
Wild- type
BAPa
Control
Restrained
Plasma ACTH
3
2
1
0
ng/ml plasma
B
AB
AB
A
Wild-type
BAPa
Treatment: NS
Genotype: NS
Interaction: P<0.05
Plasma Corticosterone
150
100
50
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ng/ml
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B
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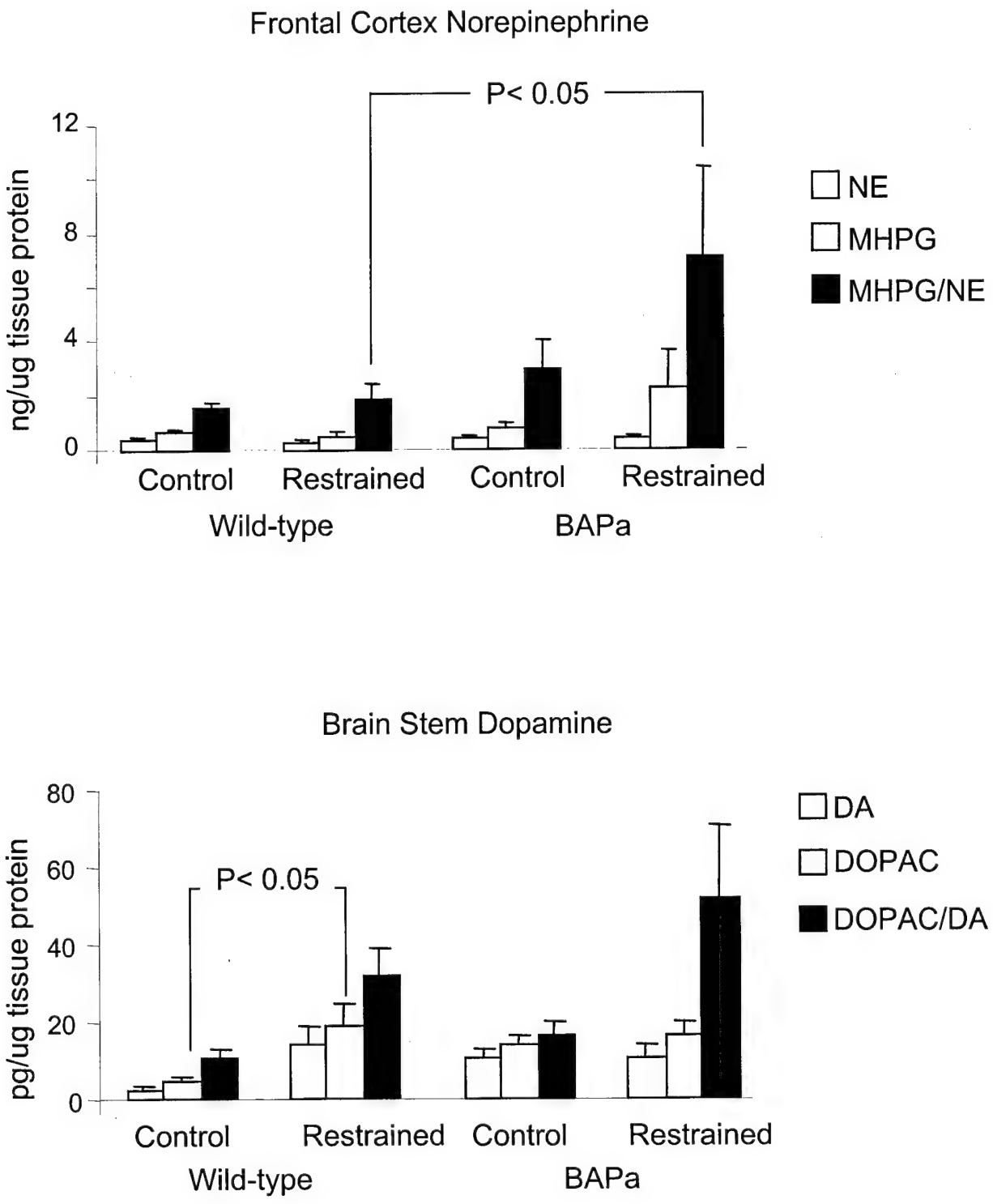
Figure 5


Frontal Cortex Norepinephrine
P< 0.05
ng/ug tissue protein
12
8
4
0
NE
MHPG
MHPG/NE
Control
Restrained
Control
Restrained
Wild-type
BAPa
Brain Stem Dopamine
pg/ug tissue protein
80
60
40
20
0
P< 0.05
DA
DOPAC
DOPAC/DA
Control
Restrained
Control
Restrained
Wild-type
BAPa

The Effects of Repeated Restraint on the Response to a Subsequent Stressor in Rats

Petra Watson, Ruth Harris, Tiffany Mitchell, Jun Zhou

Introduction

Repeated restraint causes a sustained reduction in body weight that is not associated with any maintained disruption of the CRF or catecholamine systems (3). It is possible that the change in body weight is an end point indicator of a maintained change in sensitivity to low level stressors. Individuals who have been exposed to a severe stress or a trauma show abnormal responses to subsequent stressors and it is possible that the rats exposed to repeated restraint experience a similar change in stress sensitivity. The objective of this experiment was to determine whether rats that had been exposed to repeated restraint showed increased endocrine or neurochemical responses to a subsequent stressor.

Methods

Thirty-two male Sprague Dawley rats were housed individually with free access to diet containing 41% kcal fat. After 10 days adaptation to the diet the rats were divided into two weight-matched groups. One group was restrained for 3 hours a day on each of three days (S) and the other groups were non-restrained controls (C). The rats were allowed one week to recover from restraint, were exposed to a second stressor and were decapitated immediately. Half of the rats in each of the S and C groups were restrained for 30 minutes in ice-cold water (SS and CS) the other half were non-stressed controls (SC and CC). When the rats were killed the brain was snap-frozen for measurement of catecholamine metabolism in the hypothalamus and frontal cortex. Blood was collected for measurement of glucose, insulin, IL-6, corticosterone and leptin concentrations. Fat pads were weighed as an index of adiposity of the rats.

Results

The daily body weights of the rats are shown in Figure 1. Restrained rats lost weight and ate less during stress. Once stress ended food intake returned to control levels but the rats did not recover their body weight. There were no differences in serum glucose or IL-6 concentrations at the end of the experiment (data not shown). Corticosterone was significantly higher in SC than CC rats but was low in all of the rats that had been exposed to cold water restraint. This suggests that the stressor was so severe that the adrenal gland had been depleted for glucocorticoid. Insulin was lower but leptin was higher in rats that were exposed to cold water restraint, compared with their respective controls (see Figure 2).

There were no differences in norepinephrine contents of the hypothalamus or frontal cortex. The second stress significantly increased hypothalamic serotonin and serotonin metabolism (5-HIAA/5-HT) and dopamine and dopamine metabolism (DA/DOPAC) (Figure 3). DOPAC, a dopamine metabolite, was significantly higher in rats that had been exposed to repeated restraint, independent of their exposure to a second stressor. In the frontal cortex stress significantly increased DA, DOPAC and 5-HT (Figure 4). Exposure to repeated restraint caused a significant increase in 5-HT and 5-HIAA, independent of the second stressor.

Conclusions

The stress of restraint in cold water was too extreme to allow us to determine whether rats that previously been restrained had an increased response to a second stress. All of the animals

exposed to this stressor had reduced levels of corticosterone, compared with controls, probably because the adrenal glands had been depleted of their stores. The stress significantly increased the release of dopamine and serotonin in both the hypothalamus, which mediates endocrine responses to stress, and the frontal cortex which mediates anxiety-type behavior. In the rats that were not exposed to the second stressor, being moved into the same room as restrained rats caused a greater stress response in animals that had been exposed to repeated restraint than in rats that had not been stressed before. These results suggest that repeated restraint may be a model for Post Traumatic Stress Disorder. This has to be confirmed using a milder stress as the second stress.

Chronic Effects of Repeated Restraint on Body Weight in Rats

Steven Leshin, Ruth Harris, Tiffany Mitchell

Introduction

Repeated restraint stress causes weight loss that is not recovered during the post-stress period. In a previous study restrained rats were followed for 40 days after the end of stress and did not return to the weight of control rats, but it was not clear whether the change in weight of the rats was permanent, or not. Therefore, one of the objectives of the current study was to address this issue by following the body weights of rats that had been exposed to repeated restraint for an extended period of time once stress had ended.

Recently we have been trying to determine whether repeated restraint is a model for post-traumatic stress disorder (PTSD). Many of the post-stress changes in restrained rats are similar to those reported for individuals who have PTSD. In one animal study that was completed last summer we found a suggestion of increased neuropeptide and hormonal responses to stress in rats that had been subjected to repeated restraint and were then exposed to a second severe stress of restraint in ice-cold water. In a more recent study, we did not find any evidence for increased endocrine responsiveness to a very mild stress of 2 minute of swimming in rats that had been subjected to repeated restraint. Therefore, the second objective of this experiment was to determine whether there was any evidence for an increased behavioral response to subsequent stressors in rats that had been exposed to repeated restraint.

Methods

Male Sprague-Dawley rats (300-324 g), obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), were housed in individual hanging cages, in a room maintained at 21-24 °C, on a 12 hour light/dark photoperiod (lights on at 7:00 am). Rats had ad libitum access to pelleted chow (Purina Rat Diet 5012) and water, except during experimental treatments. A week following delivery of animals, body weights and food intakes, corrected for spillage, were measured each morning.

After a week of baseline measurements, rats were divided into two weight matched groups. In the morning on three consecutive days (d 9, 10, 11) rats were moved to another room and either restrained for 3 hours in clear plastic cylinders, (6.4 cm diameter, Broome Restrainers), "restraint" group, or were placed in novel plastic cages with hardwood chips, "no restraint" group.

On days 30 and 31 of the experiment (days 21 and 22 from the initial restraint stress) half of the rats were subjected to a second, novel stress followed immediately by measurement of anxiety-type behavior in a Defensive Withdrawal test, as described below. The rats within each of the restraint and non-restraint groups were divided into two weight-matched subgroups. One

subgroup from each treatment was subjected to the second stress of 5 minutes swimming in ice-cold (6°C) water. Thus there were four experimental groups: Restraint/No Swim; Restraint/Swim; No Restraint/No Swim; No Restraint/Swim. The second stress and subsequent behavioral testing were performed between 9:30 am and 1:00 p.m. At the end of the swim the rats were towel-dried as they were carried to the site of behavioral testing. Rats in the No Swim groups were placed in shoe-box cages for 5 minutes and were also towel-dried with a damp towel as they were carried to the behavior room.

Daily measures of food intake and body weight were continued for 5 days after this second stressor then the rats were weighed once each week until Day 95 of the study. At the end of the experiment the rats in the restraint and control groups were each subdivided into two weight matched groups. One was exposed to 30 minutes of restraint and the other was a non-restrained control group, housed in a shoe-box cage, without food or water, in the same room as restrained rats for the period of restraint. The rats were decapitated as soon as the stress ended. Blood was collected for measurement of corticosterone (Corticosterone RIA: ICN Diagnostics, CA), adrenal glands and thymus were weighed.

Defensive Withdrawal Test

The apparatus consisted of a brightly-lit open field with a white floor (80-cm square) and 30-cm high black walls. Tape was used to divide the floor of the open field into 20-cm squares. The 12 outer squares were considered the outer zone and the four central squares were considered the center zone. A sheet of Plexiglas covered the floor and tape. A black cylinder (9.5 cm diameter, 15 cm long) with one open end was positioned mid-way along one of the walls. At the start of the test, rats were placed inside the cylinder and behavior was recorded for 5 minutes. The apparatus was cleaned with acetone between each test. Behaviors were recorded on videotape through an overhead CCD monochrome camera (Panasonic WV-BP330) equipped with a zoom lens (Computar T2Z3514CS Varifocal Lens). The following behaviors were evaluated: time in and time out of the black cylinder, number of entries into the cylinder, time and distance traveled in the outer zone, time and distance traveled in the center zone, time and number of grooming and rearing behaviors. At the end of behavioral testing, rats were returned to their "home" cages and food intake was monitored daily for another week. Body weight was recorded daily for one week and then on a weekly basis.

Statistical Analysis

Daily body weight and food intake were analyzed for the effects of restraint by repeated measures ANOVA. Body weight obtained on day 9, before restraint was started, was used as a covariant in this analysis. Behaviors, were analyzed as a nested factorial with day of behavior test, and cold swim stress as factors with cold swim nested within restraint to do the restriction on randomization

Results

The body weights of rats exposed to repeated restraint and their controls are shown in Figure 5. The restraint caused a significant loss of weight and the stressed rats weighed less than their controls from the second day of restraint to the end of the study. The difference remained significant on Day 94 ($P<0.05$). Food intakes are shown in Figure 6A. The restrained rats ate significantly less than controls on the days that they were restrained but intake returned to control levels after stress ended.

Analysis of behaviors following swim stress, revealed that the cold swim stress increased anxiety-type behaviors, dramatically reducing exploratory behaviors (Table 1). Rats that had been made to swim tended to stay in the dark cylinder and traveled very little when they entered the open field. There were no differences in stress-induced behaviors of rats that had previously been restrained and those that had been non-restrained controls.

Table 1: Defensive Withdrawal Test

Behavior	No Restraint No Swim	Restraint No Swim	No Restraint Swim	Restraint Swim	Statistical Analysis
Latency to exit chamber	28 ± 10	18 ± 6	40 ± 27	16 ± 7	
Number of entries into chamber	2.3 ± 0.6	1.0 ± 0.3	0.1 ± 0.1	0.3 ± 0.2	Swim: P<0.052
Total time in chamber (s)	135 ± 36	174 ± 38	250 ± 31	215 ± 38	Swim: P<0.038
Total time in outer zone (s)	172 ± 38	122 ± 37	50 ± 31	85 ± 38	Swim: P<0.034
Total distance traveled (cm)	457 ± 103	467 ± 148	10 ± 11	28 ± 12	Swim: P<0.0001
Grooming events	2	1	0	0	
Unassisted rears	0.3 ± 0.2	0.7 ± 0.4	0	0	
Assisted rears	7.5 ± 1.9	4.4 ± 1.4	0	0.4 ± 0.3	

At the end of the experiment there were no significant differences in the adrenal or thymus weights of the rats (Table 2). Corticosterone, measured immediately after 30 minutes of restraint was significantly lower in rats that had been exposed to repeated restraint at the start of the study, compared with those that had not been restrained before (Figure 6B).

	Control-Control	*Control-Restrained*	*Restrained - Control*	*Restrained-Restrained*
Adrenals (mg)	71 ± 2	77 ± 3	75 ± 4	69 ± 3
Thymus (mg)	243 ± 21	231 ± 17	213 ± 8	219 ± 16

Table 2: Data are means ± sem for groups of 10 rats killed on Day 95 of the experiment. Restrained-Controls and Restrained-Restrained rats had been exposed to repeated restraint on Days 9-11 of the study. Control-Restrained and Restrained-Restrained rats had been restrained for 30 minutes immediately before being killed. There were no significant differences between groups for either of these measures.

Conclusions

The results of this experiment demonstrate that repeated restraint stress has a chronic effect on body weight as restrained rats weighed more than controls 83 days after the end of restraint. Although restrained rats weighed less, both groups were gaining weight at a similar

rate which implies that the stress causes a "resetting" of an equilibrium that causes the rats to maintain a smaller body size.

The second objective of the study was to determine whether repeated restraint caused rats to be more responsive to a second stressor. The results from a behavioral test performed after 2 minutes of swimming in ice-cold water suggested that there were no differences in response between rats that had previously been restrained and those that were non-restrained controls. The values for corticosterone measured after 30 minutes of restraint at the end of the experiment, however, showed a lower level of corticosterone in previously stressed rats. If they were more responsive to stress it would be anticipated that the corticosterone concentrations would be higher. An experiment is now in progress to determine whether the lower level of corticosterone is due to a blunted hormonal response to stress or if the timing of the response is different in the two groups of rats. The single time point measure made in this experiment makes it impossible to differentiate between these two alternative explanations for the difference between restrained and control rats.

The Effect of Repeated Restraint on Energy Expenditure in Rats

Kimberley Freeman, Emily Kelso, Tiffany Mitchell, Bill Flatt,

Introduction

Previous experiments have shown that rats exposed to repeated restraint stress (3 hours of restraint on each of 3 consecutive days) causes a temporary inhibition of food intake but a sustained reduction in body weight of the rats. The rats lose weight during restraint and then start to grow at the same rate as control rats but do not replace the weight that was lost during stress. We have demonstrated that although the restraint does not induce fever, there is significant hyperthermia during the period of restraint. The objective of this experiment was to determine whether weight loss in the restrained rats was associated with a significant elevation in energy expenditure and whether there was any indication of hypermetabolism in the rats during the period following stress. Increased expenditure without a simultaneous increase in energy intake could contribute to the maintenance of a reduced body weight in stressed rats.

Methods

Twelve male Sprague Dawley rats, weighing approximately 350 g, were housed individually in the rodent calorimeter maintained by Dr. Flatt in the Department of Foods and Nutrition, UGA. The calorimetry laboratory is maintained at 25°C with lights on 12 hours a day from 7.00 a.m. A computer-controlled, open circuit calorimetry system (Oxymax, Columbus Instruments, Columbus, OH) was used. The system contains 12 cages, each with a food hopper and water bottle and wire floor. Oxygen consumption, carbon dioxide production, respiratory quotient (RQ) and heat production of each rat was measured at 19.5-minute intervals. Airflow was controlled and measured using a mass flowmeter for each chamber. Gas composition of incoming outdoor air and exhaust gas were measured using an infrared gas analyzer for carbon dioxide and an electrochemical oxygen sensor battery system. The gas analyzers were calibrated daily using cylinders of primary gas standard mixtures with known concentrations of CO_2, O_2 and N_2. RQ was calculated as CO_2 production/O_2 consumption (liters). Heat production (HP) was calculated based on the Brouwer equations. HP (kcal) = 3.820 O_2 consumption (liters) + 1.150 CO_2 production (liters).

Daily body weights and food intakes, corrected for spillage, were measured between 8.00 and 9.00 a.m. each day. Feces were collected from each animal for determination of digestible energy. The rats were adapted to the calorimeter for 5 days. They were divided into two weight matched groups and one group was restrained for 3 hours a day for 3 days. Stressed rats were placed in Plexiglas restraining tubes inside the calorimetry cages so that heat production during restraint could be measured. The controls were housed without food or water for the period of restraint. The daily energy expenditure of the rats was monitored for a week after the end of restraint and then the rats were removed from the calorimeter.

Results

This study finished last week, therefore, data analysis is not complete. The results for 24-hour measurements have been plotted but have not been analyzed for statistically significant differences. The body weights of the rats are shown in Figure 7. The rats that were exposed to repeated restraint lost weight during stress and had not returned to the weight of control rats by the end of the experiment. Food intake (Figure 8) was inhibited on the days of restraint but there were no differences in intake of control and stressed rats once stress ended. The respiratory quotient (RQ) is shown in Figure 9. The RQ falls when fatty acids are oxidized. It is clear from Figure 9 that stress induced fatty acid oxidation on the days of restraint but that carbohydrate oxidation was restored once stress ended. The effects of restraint on 24-hour heat production are shown on Figure 10 and it appears that the weight loss associated with stress is not due to a large change in daily expenditure. The daily data will be analyzed in more detail to determine whether there are any changes in the diurnal pattern of expenditure in rats that have been restrained.

Conclusions

In a previous study we had found that the body temperature of rats exposed to 3 hours of restraint was significantly elevated by approximately 2 ^{o}C during the period of restraint and then returned to control values within an hour of the end of restraint. This increase in temperature is indicative of an increased rate of heat production. Therefore, the objective of the current study was to determine whether repeated restraint induced a significant and sustained elevation of heat production that could contribute to the initial weight loss and sustained reduced body weight of stressed rats.

The results suggest that there is not a sustained change in 24 hour energy expenditure of stressed rats, when the data is expressed per rat (Figure 10) or per unit metabolic body size (weight $^{0.75}$: data not shown). The daily data will be examined to determine whether there was a measurable increase in heat production during the 3 hours of restraint and, if so, how long the hypermetabolism was maintained. It is possible that the increased body temperature, and the assumed increased energy expenditure, during restraint are stimulated by activation of the sympathetic nervous system or release of inflammatory cytokines such as IL-1 or IL-6. Both of these acute responses to stress are of short duration and would have a transient effect on energy expenditure.

Stress Responses in Mice Injected with □MSH

Jun Zhou, Mingxai Shi, Ruth Harris

Introduction

We previously found that mice over-expressing Agouti protein, and endogenous antagonist of melanocortin receptors, exhibit an exaggerated behavioral response. Because the transgenic mice over-express agouti protein throughout development the increased stress-responsiveness may represent a compensatory response to the loss of functionality of the melanocortin system. The objective of this experiment was to manipulate the melanocortin system during acute stress in order to determine whether this system is involved in the behavioral response to stress under normal conditions. The initial intention was to treat mice with the melanocortin receptor antagonist AgRP immediately before exposure to stress. This reagent was not available therefore we treated mice with a-melanocortin stimulating hormone (□MSH), an agoinist of melanocortin receptors, immediately before exposure to each stressor. If antagonism of the system increases stress responsiveness then agonism would be expected to reduce the stress-responsiveness of the animals.

Methods

Forty nine male, adult C57BL/6J mice were obtained from Jackson Laboratories and were housed individually in a temperature controlled room at 73°F, with lights on 12 hours a day from 7.00 a.m. They all had free access to water and to mouse chow and were adapted to handling before the experiment started.

The mice were divided into two groups. Saline injected (0.2 ml i.p.) and □MSH injected (160 ug in 0.2 ml saline, i.p.). Thirty minutes later mice in each group were subdivided into restrained (saline RS and □MSH RS) or control, non-restrained Mice (Saline C or □MSH C). The mice were placed in plastic restraining tubes for 12 minutes and the non-restrained mice were moved into the same room without access to food or water for the period of restraint. Immediately after the end of stress anxiety behavior was tested in a Light-Dark box, described below. Three days later, daily measures of food intake and body weight were initiated. After 10 days of baseline measurements, half of the mice were subjected to repeated restraint stress. For repeated restraint the mice received the same injections of either saline or □MSH and remained in the previously assigned treatment groups. Restraint was extended to 2 hours a day and repeated in the morning on each of 3 consecutive days. Thirty minutes after starting the first restraint stress, a small amount of blood was collected from each mouse by tail-bleeding to measure corticosterone (Rat Corticosterone RIA: ICN Diagnostics, CA). At the end of the last restraint, rectal temperatures were measured. The measurements of daily food intake and body weight were continued for another 10 days after the last restraint stress.

Light-Dark Box

The apparatus consisted of two polyvinyl chloride boxes (23.5 x 23.5 x 23.5 inches). One was dark and enclosed with black walls, floor and lid. The light box had white walls and floor and was illuminated by a 75 W lamp placed 3 inches above the box. The dark and light compartments were connected by a semicircular hole (9 cm high by 5 cm wide) that allowed mice to move between the two areas. Each mouse was tested for 5 minutes immediately after the eend of stress and the following behaviors were measured: Latency to escape the dark box, number of exits from the dark box, total time spent in the box.

Statistical Analysis

Serum corticosterone and body temperature were analyzed by two-way analysis of variance. Food intakes and body weights were analyzed by repeated measures analysis of

variance with Day as the repeated measure. SAS System version 6.12 was used for computations. Data are represented as means ± sem

Results

The results of the anxiety behavior test are shown in Figure 11. Two way ANOVA indicated a significant effect of stress on latency to first escape from the dark box, number of exits from the dark box and total time spent in the light box. There was no effect of □MSH on any of these measures. Serum corticosterone, measured after 30 minutes of stress on the first day of repeated restraint is shown in Figure 12. □MSH treated mice had higher levels of corticosterone than the saline injected mice ($P<0.06$). There was no significant effects of □MSH on rectal temperatures although restraint stress caused a significant (Figure 13: $P<0.01$) reduction in temperatures of the mice. Change in body weight after repeated restraint is shown in Figure 14. Repeated restraint caused weight loss in all mice but there was no effect of □MSH. Stress caused a significant inhibition of food intakes of the days of restraint but intake returned to control levels once stress ended and □MSH had no effect on the response (Figure 15).

Conclusions

The only effect of □MSH on the stress response of wild type mice was to increase stress-induced corticosterone release, which confirmed that the injection had activated the melanocortin Type 2 receptor (MCR-2), which is the ACTH receptor. □ MSH did not modify either anxiety-type behavior following a brief period of restraint stress and did not change the amount of weight loss in response to repeated restraint. Based on the results from experiments with transgenic mice that over-express and antagonist of melanocortin receptors, we had anticipated that □MSH would blunt the stress responsiveness of wild-type mice. The results of this experiment suggest that the increased stress-responsiveness of transgenic mice is due to developmental compensation for inactivity of the melanocortin system and it remains to be determined what role is played by the melanocortin system in behavioral and neurochemical responses to stress under normal conditions.

Setting up the Method of Double-color Fluorescence *in situ* hybridization

Xiaochun Xi, Jun Zhou and Roy Martin

Introduction

In situ hybridization is a powerful tool for the localization and quantification of gene expression in cells. However, it is also a laborious and time–consuming method. We demonstrate a novel double color fluorescence in situ hybridization (double-color FISH) to detect two genes expression at same time in the same tissue section. In order to set up the method of double-color FISH, two kinds of dopamine transporter antisense RNA probes were used; one was digoxigenin-labeled DAT probe; the other one was fluorescein-labeled DAT probe. In this report, I will describe how to use fluorescein and digoxigenin-UTP labeled Dopamine transporter antisense RNA probes, followed by TSA amplification system, to directly detect DAT gene expression in the same tissue section. The establishment of this method will allow us to detect multiple target gene expression in the cell.

Material and methods

Probes preparation: A 508 bp DNA fragment (position 38-545 of the rat DAT cDNA) was amplified by RT-PCR and cloned into PCR II. The resulting plasmid was linearized and transcribed with T7 or SP6 RNA polymerase to generate sense or antisense RNA probes, respectively. One set of DAT-probes (sense and antisense) were labeled by incorporation of digoxigenin (Dig)-UTP, while another set of DAT-probes was labeled by incorporation of fluorescein (Flu)-UTP using MAXIscript T3/T7 kit (Ambion). The concentration of the probe was about 0.5 ug /ul each.

In situ hybridization: Rodent brains were collected, frozen immediately on dry ice and stored at -80°C until use. Frozen sections (16 um thick) were collected onto Supreplus electrostatically coated slides from -5.4 mm to –6.0 mm relative to Bregma. The sections were fixed in 4% parafomaldehyde, acetylated, then dehyrated prior to hybridization. Sections were hybridized in two antisence probes, two sense probes, or no probe hybridization mixture (1:500; vol: vol) at 60°C for 15 hours in a humid chamber. Slides were then treated with Rnase A and washed with high stringency. The cells were visualized by a combined immunocytochemical reaction using anti-fluorescein-hrp and anti-dig-hrp, followed by fluorescein and cyanine 3 Tyramide signal amplification (TSA System). Photomicrograph was analyzed using a fluorescent microscope connected to a video recorder to digitize the images. The picture showed was taken by Dr.Hans Berthoud. The detailed procedure will be shown on separate pages. The title is Double-color Fluorescent In situ hybridization protocol.

Results (see figure 16 and 17)

The pictures clearly showed that both probes (red and green color) worked well. Using antisense probes, we could detect DAT mRNA expression in the substantia nigra of the midbrain, which has been proved by previous study; while sense probes and non-probe control showed no signals in the same area. In conclusion, we have successfully established the double-color FISH method. With this method we can detect the multiple gene mRNA expression in the same tissue section.

Acknowledgements.

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Cloning of Glucose transporter type 2 and Preproinsulin partial cDNAs from Rat

Xiaochun Xi, Jun Zhou and Roy Martin

Introduction

To study the interaction of short term and long term signals on brain's sensing of glucose, in term of feeding behavior, we are interested in some particular gene's expressing in the brain, such as Glucose transporter type 2 and Preproinsulin. These partial cDNA of genes have been cloned.

Methods

I Amplification cDNAs by RT-PCR

First-strand cDNAs synthesis by Reverse Transcription (RT).

Total RNAs were isolated from liver of rat using Trizol reagent (Gibco). Oligo(dT)15 was used . The RT reaction contained 1X RT buffer, 5mM MgCl2, 2ug of total RNAs, 100 uM dNTPs, 0.5 ug Oligo(dT)15 primer, 20 units of AMV reverse transcriptase in 20 ul total volume. The first-strand cDNAs were synthesized at 42°C for 30 minutes then the reaction was heated at 95°C for 5 minutes.

PCR amplification of partial cDNAs.

Aliquots of the first-strand cDNA were used as templates for specific amplification of Glucose transporter type 2 and Preproinsulin partial cDNAs. The sequence of primers was based on cDNA sequence publised in NCBI. The following primers were designed to amplify the partial open reading frame of the cDNAs:

Glut-F primer:	5'CTTCCTCGCCTGGTTCTACTGA
Glut-R primer:	5'CGAGCGTTTAACCTTACAATATAC
INS-F primer:	5'CCTGCCCAGGCTTTTGTCAAAC
INS-R primer:	5'AGTTGCAGTAGTTCTCCAGTTG

These primers were synthesized by Gene Lab at Louisiana State University. The PCR reaction contained 1X RT buffer, 2mM MgCl2, 10 ul of first-round RT reaction, 100 uM dNTPs, 500 nM primers, 2.5 units of Taq polymerase in 100 ul total volume. The partial cDNA was amplified for 35 cycles using the following conditions: 94°C for 40 seconds, 60°C for 1 minute and 72°C for 1minute. An aliquot of PCR reaction subjected to electrophoresis revealed a 395 bp product for Glut-2, a 771 bp product for INS-2 and a 271 bp product for INS-1.

2. Cloning of partial cDNAs into pCR II

The PCR products were cloned into pCR II (Invitrogen) according to the manufacturer's protocol. The construct was transformed into *E.coli* DH5α cells and the presence of the insert was confirmed by restriction analysis of minipreparations of plasmid DNA cut with *Eco*R I. The partial cDNAs were sequenced by Gene Lab at LSU.

Results

The isolated partial cDNA was 395 base pairs long and matched the sequence of the Rat Glut-2 cDNA published in NCBI (NM_012879) perfectly. The isolated partial cDNA was 771 base pairs long and matched the sequence of the Rat INS-2 cDNA published in NCBI (V01243.1) perfectly. The isolated partial cDNA was 271 base pairs long and matched the sequence of the Rat INS-1 cDNA published in NCBI (NM_019129) perfectly, as shown below. The Glut-2 partial cDNAs in pCR II vector is in the reverse orientation with respect to the *Lac* gene, while INS-1 and INS-2 partial cDNA in pCR II vector are in the forward orientation with respect to the *Lac* gene.

1. Glut-2 partial cDNA clone

The nucleotide sequence of Glut-2 partial cDNA has been cloned into pCR II. The nucleotide sequences of primers for PCR are shown in bold.

CTTCCTCGCCTGGTTCTACTGAAATTGGAGAGCTTTCAACATATCAC
TGCTCTTAAAAGCACGTGACTAAGAAATCTGAAGCTACAACTGATATA
TATATATTTAAATATAATTCCATTGGAATTTTCTTAGCCACAGATCTGA
TGCTGAAGGACCTATGGCTGGCAAGGCTCACACTCAAACTGACGTCTC
TGTTACCCACCTCGGTTTCTCTCACATGTACTAGACTTTGTGTTTTGTTT
TAAACTATTTTTCTGCATTTTTATAGAGAACTCCTGAGTATGTTAAGCT
ATATTTGTAATGAAAAGCCTCTTCTTTACAGCTACCAAAATCTTCATGT
AGTGAGAGAAAACGTTTCAGTTTCTTTGAT**GTATATTGTA AGGTTAA**
ACGCTCG

2. INS-2 partial cDNA clone

The nucleotide sequence of INS-2 partial cDNA has been cloned into pCR II. The nucleotide sequences of primers for PCR are shown in bold.

CCTGCCCAGGCTTTTGTCAAACAGCACCTTTGTGGTTCTCACTTGGTGG
AAGCTCTCTACCTGGTGTGTGGGGAGCGTGGATTCTTCTACACACCCATG
TCCCGCCGCGAAGTGGAGGACCCACAAGGTAAGCTCTGCTCCTGAATTCT
ATCCCAAGTGCTAACTACCCTGTTTGTCTTTCACCCTTGAGACCTTGTAAA
TTGTGCCCTAGGTGTGGAGGGTCTCAGGCTAACCAGTGGGGGGCACATTT
CTGTGGGCAGCTAGACATATGTAAACATGGTAGCTGCCAAGAAGGAGTG
AGAATCCTTCCTTAAGTCTCCTAGGTGGTGACGGGTGGCTAGGCCCCAGG
ATAGGTACCTATTTGGGGACCCCATAGAGCACTGCACTGACTGAGGGAT
GGTAACAGGATGTGTAGGTTTTGGAGGCCCATATGTCCATTCATGACCA
GTGACTTGTCTCACAGCCATGCAACCCTTGCCTCCTGTGCTGACTTAGCA
GGGGATAAAGTGAGAGAAAGCTTGGGCTAATCAGGGGGTCGCTCAGCT
CCTCCTAACTGGATTGTCCTATGTGTCTTTGCTTCTGTGCTGCTGATGCTC
TGCCCTGTGCTGACATGACCTCCCTGGCAGTGGCACAACTGGAGCTGGG
TGGAGGCCCGGGGGCCGGTGACCTTCAGACCTTGGCACTGGAGGTGGCC
CGGCAGAAGCGCGGCATCGTGGATCAGTGCTGCACCAGCATCTGCTCTC
TCTAC**CAACTGGAGAACTACTGCAACT**

3. INS-1 partial cDNA clone

The nucleotide sequence of INS-1 partial cDNA has been cloned into pCRII. The nucleotide sequences of primers for PCR are shown in bold.

CCTGCCCAGGCTTTTGTCAAACAGCACCTTTGTGGTCCTCACCTGGTGGA
GGCTCTGTACCTGGTGTGTGGGGAACGTGGTTTCTTCTACACACCCAAGT
CCCGTCGTGAAGTGGAGGACCCGCAAGTGCCACAACTGGAGCTGGGTGGA
GGCCCGGAGGCCGGGGATCTTCAGACCTTGGCACTGGAGGTTGCCCGGCA
GAAGCGTGGCATTGTGGATCAGTGCTGCACCAGCATCTGCTCCC**TCTACC**
AACTGGAGAACTACTGCAACT

Conclusions:

These probes will be used in future studies of glucose sensing mechanisms and food intake control.

The role of dopamine system in food reward and glucoprivic feeding

Jun Zhou, Xiaochu Xi, and Roy Martin

Introduction

Feeding behavior is directly connected to energy homeostasis, and therefore, is a vital part of body weight research. Neuropeptides regulate feeding behavior by stimulating a particular brain site. However, the stimulation caused by most peptides works regardless of the drive state of animals. For instance, NPY increase food intake in satiated animals(Stanley and Leibowitz 1985) and CRF decrease food intake in the hungry animals(Jones, Kortekaas et al. 1998). Besides this type of brain neuropeptide regulation, there is another type of brain stimulation, which is only effective when the animal is in a particular energy state. For example, food serves as a "reward" stimulus only in hungry animals. The second type of brain stimulation is less studied in body weight regulation research, comparing with those brain neuropeptides. However, in most cases, it is the second type of brain stimulus "the pleasure of eating" that result in body weight deviating from the "ideal" set point in the normal, health person.

Reward is sometimes identified with the pleasure or hedonic impact of brain stimulus. Among the most of thoroughly studied of all brain substrates for reward are dopamine projections from the substantia nigra and ventral tegmentum to forebrain structures, such as nucleus accumbens and prefrontal cortex. It is well know that dopamine projections are crucial to sensorimotor function. However, investigators have concluded that dopamine projections play a role in mediating reward above and beyond sensorimotor contributions(Nader, Bechara et al. 1997; Bardo 1998).

The new research project, began this year, investigated the effect of brain reward stimulus on feeding behavior, specifically the role of dopamine system in feeding behavior in both satiated and hungry animals. Two types of food have been used in this project. One is regular chow, which should facilitate feeding in hungry but not in satiated rats. The other is palatable food (cookies), which should stimulating feeding in both satiated and hungry animals. The hungry animal was initiated by glucoprivation induced by 2-DG injection.

The glucoprivic hyperphagia induced by 2-DG administration is a well established hungry animal model. However, the area of the brain stimulated by glucoprivation coincides with areas rich in dopamine containing neurons.(Briski 1998). Furthermore, rats given dopamine depleting lesions failed to increase their food intake after 2-DG injection (Lu and Rowland 1993). However, there are no reports of direct measurement of dopamine system activity after initiation of glucoprivic hyperphagia. Therefore, the hypothesis of present study is that dopamine system is activated as the reward stimulus in both satiated and hungry animals when either cookies or regular chow act as food reward to these animals.

The activation of dopamine system could be regulated by 1) pre-synaptic vesicle's release, 2) post-synaptic receptor binding and activation, 3) the ability of dopamine transport re-uptake extracellular dopamine. In the present study, we plan to measure the dopamine content in nucleus accumbens and prefrontal cortex and dopamine transport mRNA expressing in substantia nigra and ventral tegmentum. The other indication of dopamine system activation will be further investigated, depending upon the results of this study.

Two experiments were conducted. In the first experiment, we food deprived, fed regular chow, and fed both chow and cookies to satiated and glucoprivic rats. As expected, the satiated rats did not eat regular chow, but ate significantly more cookies as this palatable food acted as reward stimulus for eating. In glucoprivic rats, their chow intake was significantly higher compare with satiated rats as the results of glucoprivic hunger. Unexpectedly, glucoprivic rats failed to choose cookies as palatable food after 2-DG administration. These rats still chose regular chow, regardless of the presence of cookies in their cages. To address this unexpected result in 2-DG injected rats, the second experiment was conducted. In the second experiment, rats were offered cookies twice before 2-DG injection to eliminate the possible effect of conditioned taste aversion caused by 2-DG injection. Moreover, cookie preferences were also tested twice on day one and day four after 2-DG injection. For both experiment 1 and 2, all the animal data were finished, as well as the serum assays of insulin, glucose, and corticosterone. The dopamine transport mRNA expression measured by in situ hybridization was finished for the experiment 1. The measurement of dopamine content in nucleus accumbens and prefrontal cortex by HPLC have not been initiated yet.

Methods

Experiment 1.

Sixty adult, male Sprague-Dawley rats, weighing 300g, were obtained from Harlan Sprague Dawley (Houston, TX) and housed in individual wire mesh cages in a humidity and temperature controlled room (22±2°C, 65-67% humidity) on a 12:12 h, light:dark cycle with light on at 07:00 AM. All rats had a free access to regular chow and tube water. Body weight was recorded daily for seven or eight days before rats were divided into two groups on the experiment day: Control, (saline, i.p. 1 ml/kg body weight) and 2-DG (2-DG 500mg/ml/kg body weight, i.p.) The rats were received injection in the experiment day between 8:00AM to10:30AM. Immediately following the injection, each group rats were subdivided into three different feeding groups: (1) food deprived, (2) feed regular chow only, and (3) fed both chow and cookies. (Vanilla Wafers, Great Value, Wal*Mart® Stores INC.) Four hours after injection, the rats were decapitated. The blood was collected for measurements of glucose (Sigma Infinity™ Glucose Reagent, Sigma Diagnostics Inc. St. Louis, MO), insulin (Rat Insulin RIA kit, Linco Research Inc. St. Louis, MO), and corticorsterone (Rat Corticosterone RIA kit, ICN Biomedicals, Inc. Costa Mesa, CA). The brain was quickly removed, frozen on the dry ice and kept on -80 °C for further dopamine transport and dopamine content measurements. For the groups of rats fed either chow or chow and cookies, Four hour of intake after injection was also recorded at the end of experiment.

Dopamine transport mRNA measurement.

Probe preparation: A 508 bp DNA fragment (position 38-545 of the rat DAT cDNA) was amplified by RT-PCR and cloned into PCR II. The resulting plasmid was linearized and transcribed with T7 or SP6 RNA polymerase to generate sense or antisense RNA probes, respectively. Probes were labeled by incorporation of digoxigenin (Dig)-UTP using MAXIscript T3/T7 kit (Ambion). The concentration of the probe was approximately 0.5 ug /ul.
In Situ Hybridization: Rat brains were collected, snap frozen on dry ice and stored at -80°C until use. Frozen sections (16 um thick) were collected onto Supreplus electrostatically coated slides from -5.4 mm to –6.0 mm relative to the bregma. The sections were fixed in 4% paraformaldehyde, acetylated, and then dehydrated prior to hybridization. Sections were hybridized with either antisense probe, sense probe, or no probe at 60°C in a humid chamber over night. Slides were then treated with RNase A and washed with high stringency. Specific labeling was visualized by incubation with an antidigoxigenin antiserum conjugated to alkaline phosphatase (Boehringer Mannheim) followed by 4-nitor blue tetrazolium chloride /5-bromo-4-chloro-3-indolyl phosphate. Sections were coverslipped with Aquamount, and then, were transformed into digital image by program SPOT Basic. The images were quantitatively analyzed by program Image-Pro Plus.
Statistically analysis: The results were first analyzed by two way ANOVA, as food treatments and injections are two variances. Then each injection group was analyzed by one way ANOVA with post-hoc Duncan's multiple range test. The SAS system version 6.12 was used for computations. Data are presented as means ± SE.

Experiment 2

Sixty adult, male Sprague-Dawley rats, weighing 300g, were obtained from Harlan Sprague Dawley (Houston, TX) and housed at the same condition as in experiment 1. As in the experiment 1, the cookies and injection 2-DG were both naïve to the rats, a conditional taste aversion could development and influence animals' choice for palatable food. In order to avoid the effect of conditional taste aversion, the cookies was offered to all rats, twice before 2-DG injection and twice after 2-DG injection, in addition on the days when 2-DG was injected, in the experiment 2. All rats were received cookies in the morning for four hours on the day 4 and day 2 before the first injection, and on the day 1 and day 4 after the first injection. For all rats, two hours and four hours food intake and preference, after food (regular chow and/or cookies) were put into the cage, were recorded on every cookies offering day.

On the first injection day, the rats were divided into two groups: control, (saline, i.p.1ml/kg) and 2-DG (2-DG i.p 500 mg/ml/kg) and received corresponding injection between 8:00AM to 10:30 AM. Immediately following the injection, the rats in each injection group was divided into four subgroups: (1) food deprived, (2) feed regular chow only, (3) fed cookies only, and (4) fed both chow and cookies. The corresponding food intakes were measured on two and four hours after injection for all rats, excepting food deprived groups. Four hours after injection, all rats were offered with regular chow only. Seven or eight days after the first injection, the rats in each group received the same treatment in terms of both injection and food offering. Four hour after the second injection, the rats were decapitated, blood was collected for the same serum assay as in experiment 1, except blood glucose was measured by glucose testing meter(Accu-Chek™ Advantage®, Roche Diagnostics Corporation. Indianapolis, IN). The brain was snap frozen and kept at -80°C for dopamine transport and dopamine content measurements.

Results

Experiment 1. The food intake and preference recorded four hours after injection were show in figure 18. Glucoprivation significantly increase chow intake in fed chow and fed both chow and cookies groups compared with saline injections. In the group of rats fed both chow and cookies, rats chose only cookies after the saline injection; but only chose chow after glucoprivation. Fig. 19 summarized the results of serum assay. Glucoprivation significantly increased serum glucose and corticosterone levels in all three feeding regime groups compared with saline injection ($P<0.01$). Serum insulin level was only increase in fed chow group rats after 2-DG injection ($P<0.01$). Dopamine transport mRNA expressing in the area of substantia nigra and ventral tegmentum, measured by in situ hybridization, was summarized in table 1. Fig.20 was also showed the in situ images from saline injection rats. Two way ANOVA analysis result indicated that there was a significant difference ($P<0.05$) by food treatment. However, the difference only existed within control rats but not in the 2-DG treated rats. More specifically, DAT mRNA expression was not difference within 2-DG injected group rats, analyzed by one way ANOVA. In three feeding treatments control animals, the food deprived rats had the lowest DAT mRNA expression, and the chow/cookie group had the highest DAT mRNA expression. The differences among three control group rats were significant. ($P< 0.05$)

Table 1. Substantia nigra and ventral tegmentum DAT mRNA expression four hour after injection. The data are mean ± SE for group of 4 to 6 rats

	Food deprived	Fed chow only	Fed both chow/cookies
Control	162±42**	240±11*	266±9
2-DG	216±40	241±19	272±18

* $P<0.1$ comparing with fed chow and cookie group within control group rats

** $P<0.05$ comparing with fed chow only group and fed both chow/ cookie group within control group rats

Experiment 2.

Body weight recorded over the whole period of experiment 2 was illustrated in fig 21. Glucoprivation did not significantly change body weight in all groups compared with saline injection group. Daily energy intake data was showed in fig. 22. The detailed statistic analysis was in processing. On cookies offering day, the two and four hours energy intake were showed in fig 23 and fig 24, respectively. Both figures illustrated the total energy intake, the percentage of energy that came from cookies, and the number of rats that chose cookies over the chow. On 2-DG injection day, the energy intakes and food preference were also measured at both two and four hours after injection. The results are shown in fig 25. Serum glucose, insulin and corticosterone measurements were illustrated on fig. 26. The blood samples were collected four hours after second injection on experiment 2. As observed in experiment 1, 2-DG injection significantly increased blood glucose and serum corticosterone levels in all four feeding regime groups compared with saline injection ($P<0.01$). The statistical analysis for serum insulin is in process.

Discussion

These preliminary results demonstrate that the glucoprivic feeding induced by 2-DG injection has produced a different food preference when compared with normal rats. The similar observation that 2-DG administration caused taste aversion, has been reported (Stephan, Smith et al. 1999). In present studies, 2-DG injected rats almost abolished palatable cookie intake in experiment 1, when both 2-DG and cookies were offered to them as a naïve stimulus. In the experiment 2, although cookies was not a naïve to the 2-DG injected rats, half of these rats' preference for cookies remained abnormal at the first time they received injection of 2-DG. The number of abnormalities was dropped to two rats at the second time when they received 2-DG injection. Considering the cookies have been offered to these rats more times before their second time 2-DG injection, this result provided evidence that 2-DG causes reversible conditional taste reversion that could be corrected.

The mechanism of changed cookies' preference in 2-DG injected rats is not clear at present time. Also, present result from gene expressing of dopamine transport indicates that dopamine system is involved with food reward stimulus in normal satiated rats. More specifically, dopamine system is activated in normal rats when they have access to food. This activation is increased while the rats have more palatable food, like cookies. In glucoprivic rats induced by 2-DG injection, their DAT levels in Substantia nigra and ventral tegmentum showed more variance, and the difference did not reach statistic significance, although the trend is the same as in control rats.

Conclusion

The palatability of food in this particular experiment is defined as the preference and amount of the food consumed. We also chose the experiment time in the morning and defined satiated rats as the rats that don't eat their regular chow because they are not hungry in the morning. However, when high palatable food, like cookies, was present to these rats in the morning when they are not hungry, they still all eat cookies, resulting the extra energy intakes. Our result showed that rats had the greatest eating rate during the first 20 minutes after exposing to cookie and basically stopped eating after two hour of exposing to cookie. For period between 20 minutes and 2 hours, they continuously consuming cookie, but the eating rate was not as great as in the first 20 minutes. Based on this data, we chose the killing time point for the next gene regulation study in the same rats. These basic food reward studies are aimed at identifying genes that control over-consumption of palatable foods. The long-range goal is to be able to control those genes in order to prevent overeating associated with food rewards mechanisms in the brain.

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Detecting Glucose-Sensing Beta-Like Cells In Rat Brain

Bing Li, Xiaochun Xi, June Zhou, Roy J Martin

Introduction

Pancreatic islet beta cells can sense peripheral glucose levels and regulate plasma glucose by secretion of insulin. GLUT2 glucose transporter (GLUT2) and glucokinase (GK) are two glucose sensors of beta cells. GLUT2 regulates glucose entry into pancreatic β cells. GK is the rate-limiting enzyme of glycolysis and regulates the rate of glucose metabolism in β cells (Matschinsky, et al. 1993). In this way, they regulate secretion of insulin and plasma glucose level. Previous studies have shown that hindbrain and hypothalamic arcuate nucleus are involved in central glucose sensing and regulation of feeding behavior. GLUT2 and GK have been found co-expressed in rat hypothalamic arcuate nucleus. GLUT2 has been shown expressed in nucleus of solitary tract (NTS) in rat hindbrain. We propose that cells involved in glucose sensing in both areas have similar characteristics to pancreatic beta cells. Like beta cells, these cells should co-express GLUT2 and GK and use them as glucose sensors. To prove this hypothesis, we need to show the distribution of GLUT2 and GK mRNA in arcuate nucleus and hindbrain. Because streptozotocin can specifically destroy pancreatic beta cells resulting in loss of expression of GLUT2 and GK, we will inject streptozotocin into cerebral ventricles and examine if the proposed beta like cells will be destroyed and lose GLUT2 and GK expression. We will compare GLUT2 and GK mRNA expression between obese and lean Zucker rats, and we propose that in obese, the hyperglycemia will increase both mRNA expressions. Last we will compare GLUT2 and GK mRNA expression between 50% food restricted and ad lib fed Sprague-Dawley rats, and we propose that food restriction leads to decreased GLUT2 and GK mRNA expression in the arcuate nucleus and hindbrain.

Methods and results

We use fluorescence in situ hybridization (FISH) to detect GLUT2 and GK mRNA expression. In order to prepare GLUT2 and GK probes, a 395bp of GLUT2 partial cDNA and a 271bp of GK partial cDNA was amplified by RT-PCR and cloned into PCRII. The resulting plasmid was linearized with restriction enzymes and transcribed with T7 or SP6 RNA polymerase to generate sense or antisense RNA probes, respectively. Using MAXIscritp T3/T7 kit (Ambion), GK probes were labeled by incorporation of digoxigenin (Dig)-UTP, and GLUT2 probes were labeled by incorporation of fluorescein (Flu)-UTP. The concentration of each probe was about 0.5ug/ul. The procedure for FISH is: after the rat is killed, whole brain is dissected quickly and stored at –70°C. Cryoslides are prepared by cutting the brain on –20°C Cryostat and

each section was 16um in thickness. Sections from Bregma -14.60 mm to –9.80mm are collected for the hindbrain. Sections from Bregma –3.30mm to –2.80 mm are collected for hypothalamic arcuate nucleus. GLUT2-Flu and GK-Dig antisense probes are used to hybridize with their respective mRNA; their sense probes are used as negative control. In immunodetection, anti-Flu-HRP (horseradish peroxidase) and anti-Dig-HRP are used to bind to fluorescein and digoxigenin, respectively. Then fluorescein tyramide (green FISH) and cyamine 3 tyramide (red FISH) are used to react with HRP and amplify the signals for GLUT2 and GK. FISH slides are kept at 4°C and away from light. Slides are examined with fluorescence microscope and images are taken with SPOT Basic software.

To detect the distribution of GLUT2 and GK mRNA expression in arcuate nucleus and hindbrain, male Sprague-Dawley rats fed chow (Labdiet 5001) were killed and whole brain was dissected. GLUT2 and GK mRNA expression was examined by FISH described above. Results: No signals for GLUT2 or GK were detected in arcuate nucleus. In hindbrain, GLUT2 and GK mRNA was found coexpressed in mlf (medial longitudinal fasciculus) area from Bregma –14.00mm to –10.00mm, and GK expression was much stronger than GLUT2 expression in the whole area (Figure 3 and 4). In area postrema and nucleus of solitary tract, GLUT2 was also detected in individual neuron cells, while GK was not co-expressed in these areas with GLUT2. The expression of GLUT2 and GK in hindbrain mlf but not in arcuate nucleus was consistent with glucoprivic study (Ritter et al, 2000), in which 5-thioglucose injections in arcuate nucleus failed to elicit feeding response, while 5-thioglucose injections in mlf area stimulated feeding response.

Discussion:

Since our preliminary data showed that GLUT2 and GK were not expressed in arcuate nucleus, in future studies, we will only examine GLUT2 and GK mRNA expression in the hindbrain. We will use three different rat models. In the first experiment, ad lib fed Sprague-Dawley rats will be cannulated in the fourth cerebral ventricle. Rats with correct cannulation will be divided into two groups. One group will be injected streptozotocin at the dose of 1mg per kg body weight in 10ul of saline through the cannula daily for 5 days; the other group will be injected saline as control. On day 6, rats will be killed and brain will be taken. In the second experiment, two groups of rats will be fed chow for two weeks. One group (n=10) will be ad libitum fed; the other group (n=10) will be fed 50% of ad-lib intake. On day 15, both groups of rats will be killed and brain will be dissected. In the third experiment, three-month old ad-lib fed obese (n=10) and lean (n=10) Zucker rats will be killed and brain will be dissected. In all three experiments, body weight will be recorded daily; blood will be collected at the time of kill and plasma glucose will be measured. For all the brains collected, cryosections of hindbrain between Bregma –14.00 mm and –10.00 mm will be made and GLUT2 and GK mRNA expression will be examined by FISH described above. We expect the outcomes to be: streptozotocin will destroy beta like cells in hindbrain and GLUT2 and GK mRNA expression will be decreased or diminished compared to ad lib control; 50% food restriction will decrease GLUT2 and GK mRNA expression in hindbrain; and in obese Zucker rats, GLUT2 and GK mRNA expression will be increased compared to leans.

Conclusions:

When completed, these studies should provide the information necessary to design intervention approaches that allow us to modify hunger and satiety signals related to the control of energy balance. For example, we will learn what cells in the brain are responsible for sensing hunger signals related to low blood glucose. This information will provide the focus necessary to study how metabolic signals are translated to feeding or satiety signals. These mechanistic studies are necessary to develop a strategic approach to altering feeding behavior in unique ways. One dietary approach to be tested next year is the use of foods that have a low glycemic load. The low glycemic index foods are ones that release glucose at a slow rate so that blood glucose levels are maintained for extended periods of time. It is hypothesized that foods with a low glycemic index will produce a delay in hunger signals associated with hypoglycemia and reduce overall food intake and body weight.

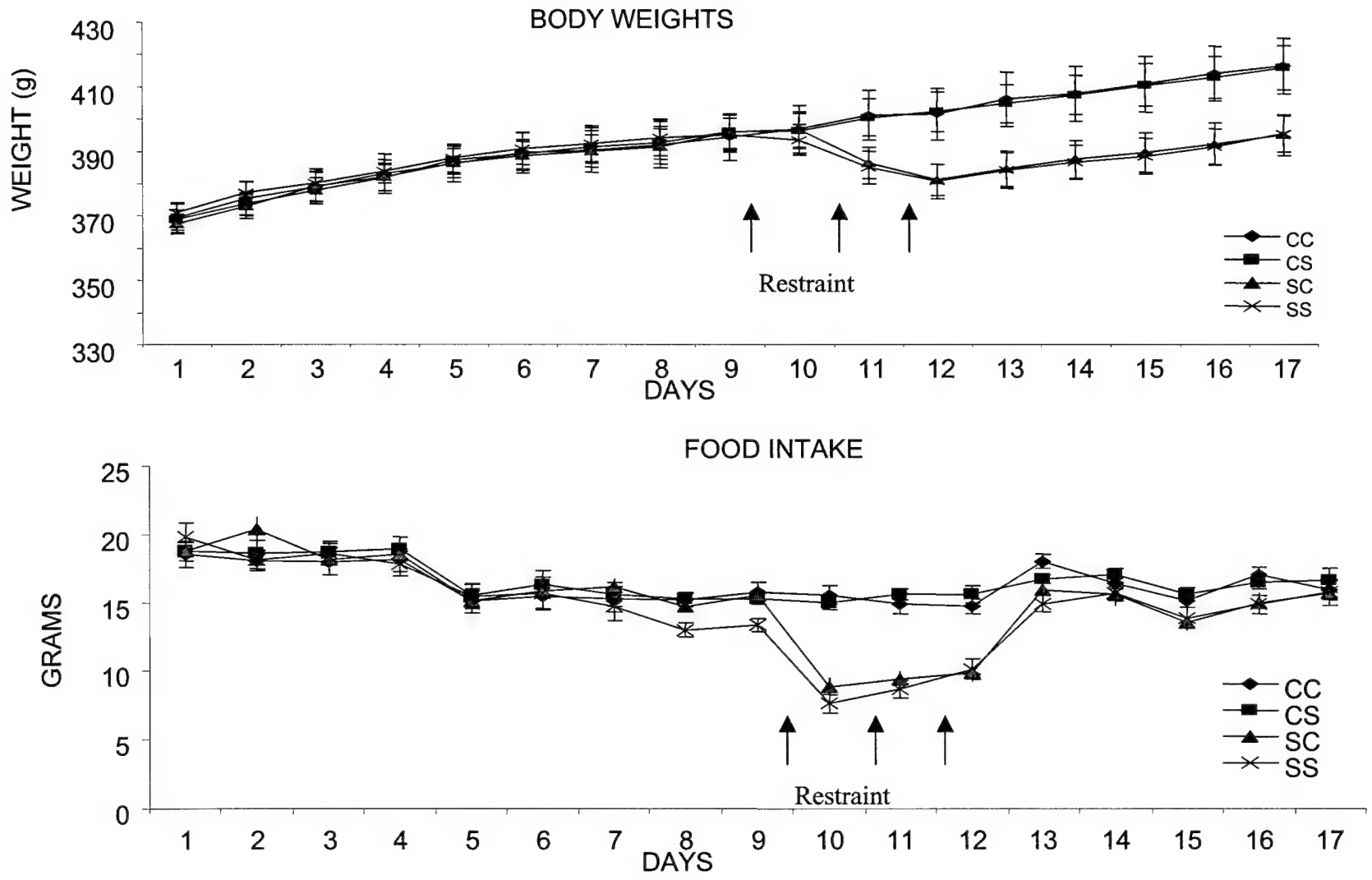


Figure 1: Food intake and body weights of rats exposed to repeated restraint and then subjected to a second stress at the end of the experiment

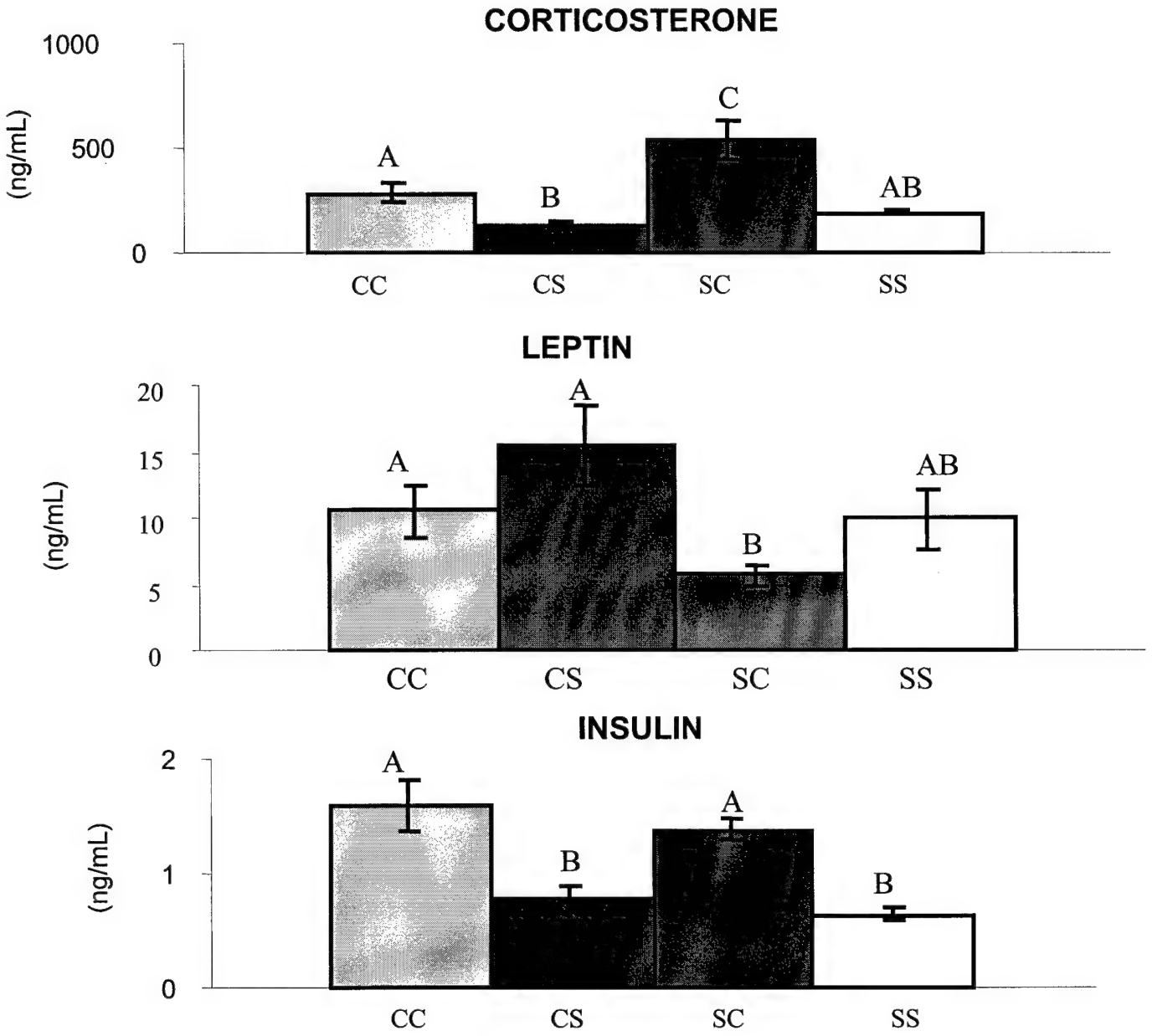


Figure 2: Serum hormones in rats exposed to repeated restraint and then subjected to a second stress at the end of the experiment

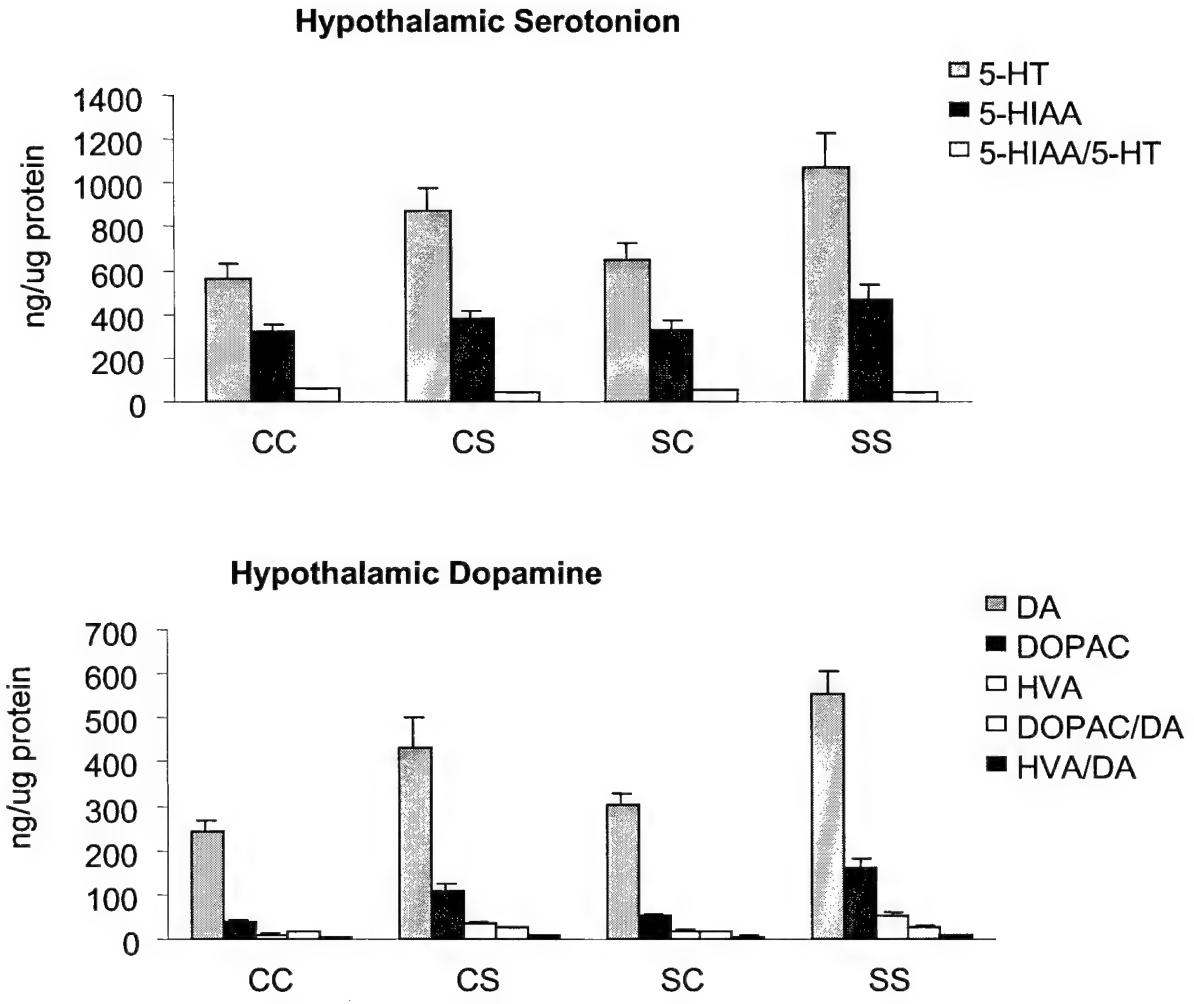


Figure 3: Monoamine metabolites in the hypothalamus of rats exposed to repeated restraint and then subjected to a second stress at the end of the experiment

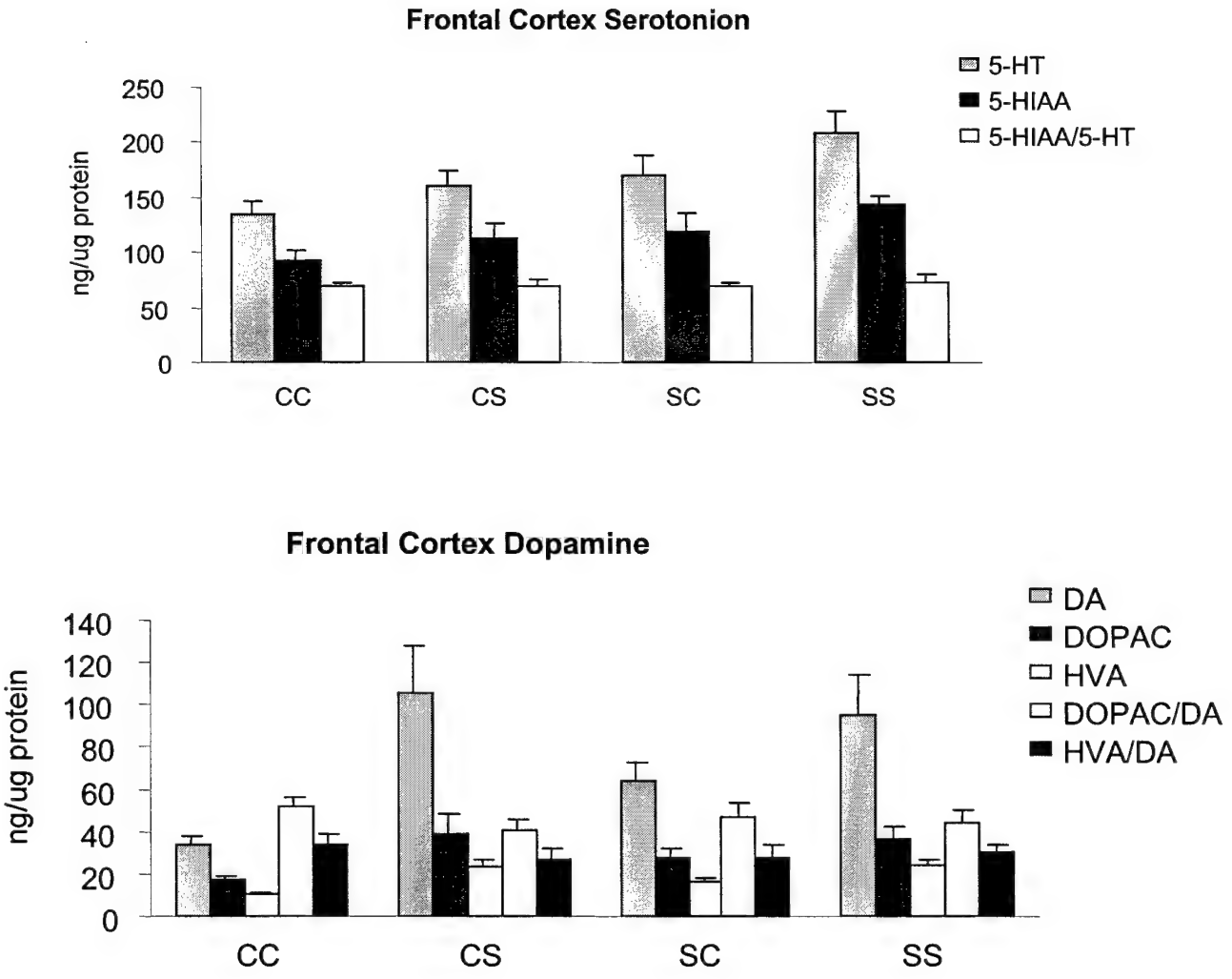


Figure 4: Monoamine metabolites in the frontal cortex of rats exposed to repeated restraint and then subjected to a second stress at the end of the experiment

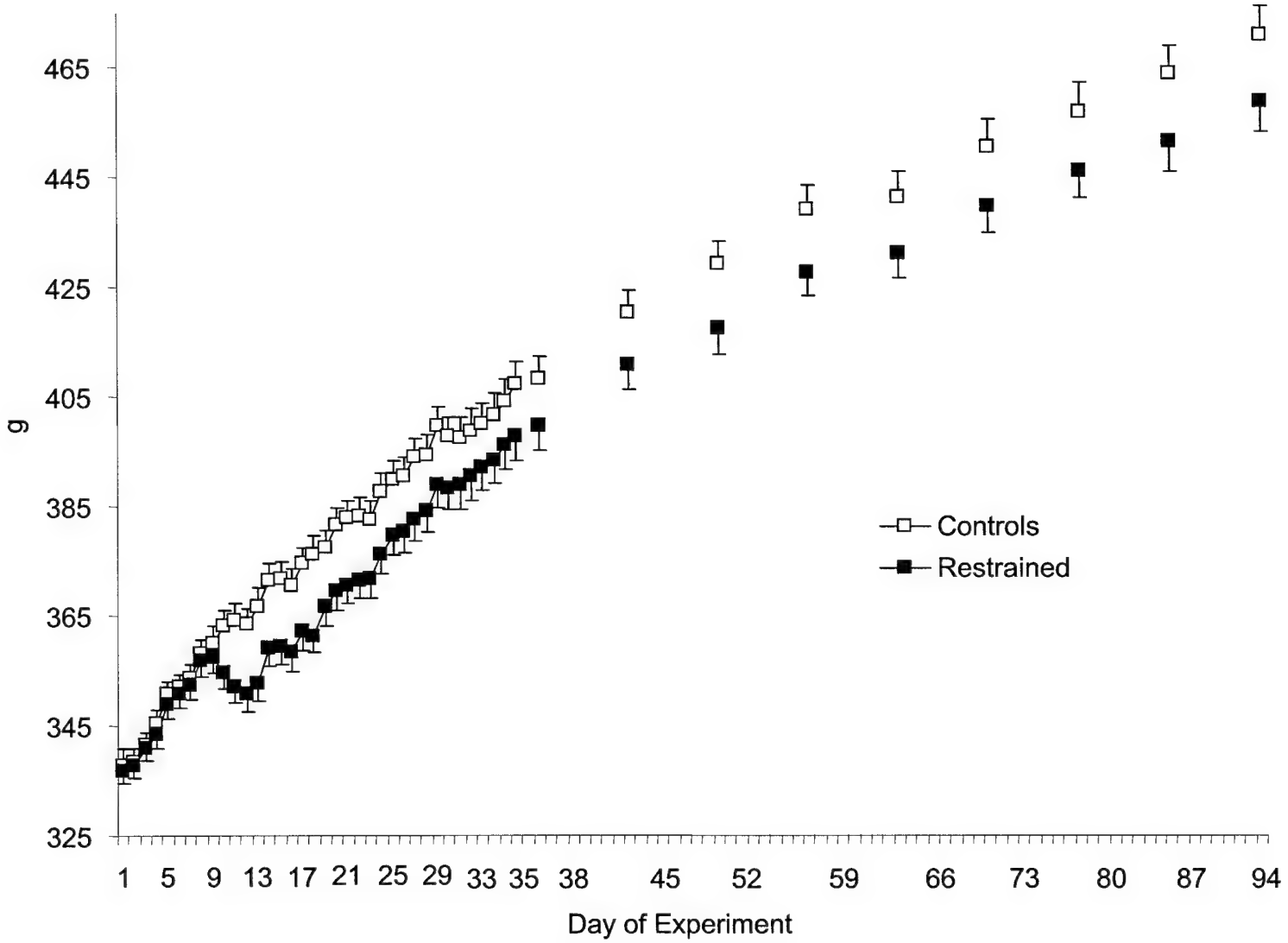


Figure 5: Body weights of rats exposed to repeated restraint on days 9 to 11 and their non-restrained controls. Data are means $\pm$ sem for groups of 20 rats. The weights of the two groups of rats was significantly different from the second day of restraint to the end of the experiment.

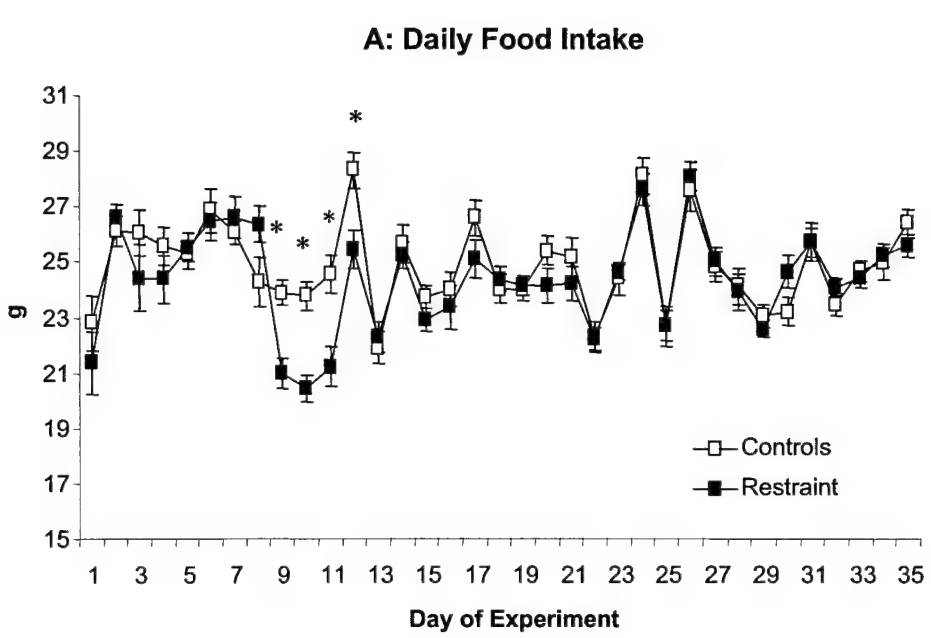


Figure 6A: Daily food intake of rats exposed to repeated restraint on Days 9 to 11. Data are means + sem for groups of 20 rats. An asterisk indicates days on which intakes were significantly different.

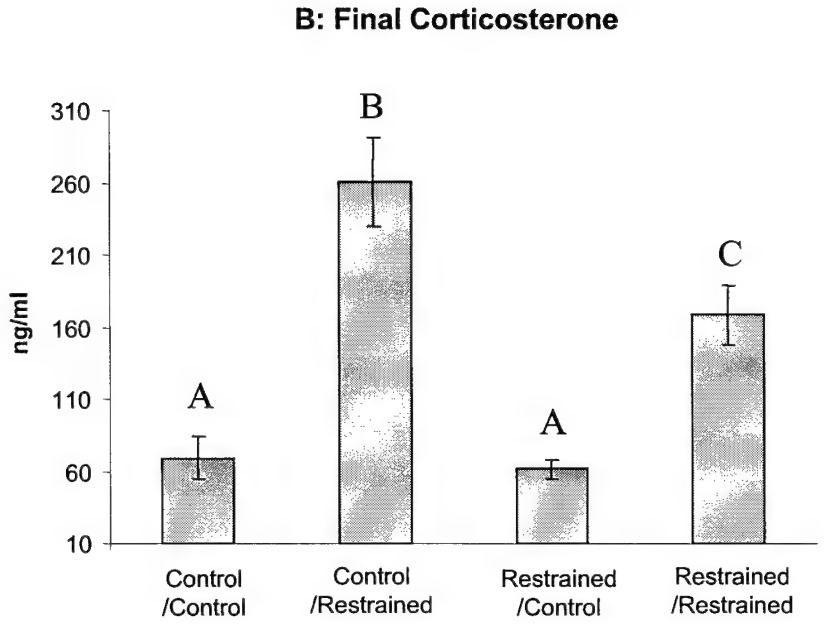


Figure 6B: Serum corticosterone measured on Day 95 of the experiment. Data are means + sem for groups of 10 rats. Control-restrained and Restrained-Restrained rats were restrained for 30 minutes immediately before they were killed. Values that do not share a common superscript are significantly different at $P<0.05$.

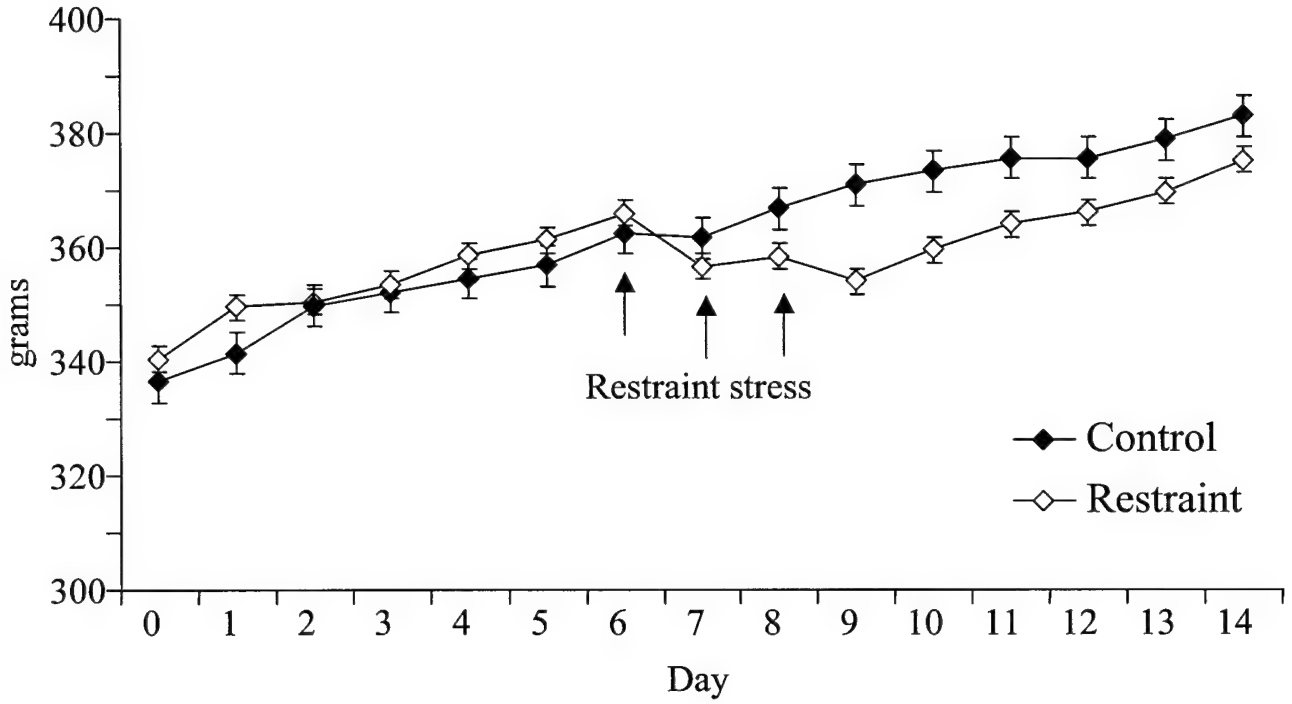


Figure 7: The effects of repeated restraint stress on body weight of rats housed in calorimetry chambers

Data are means ± sem for groups of 6 rats

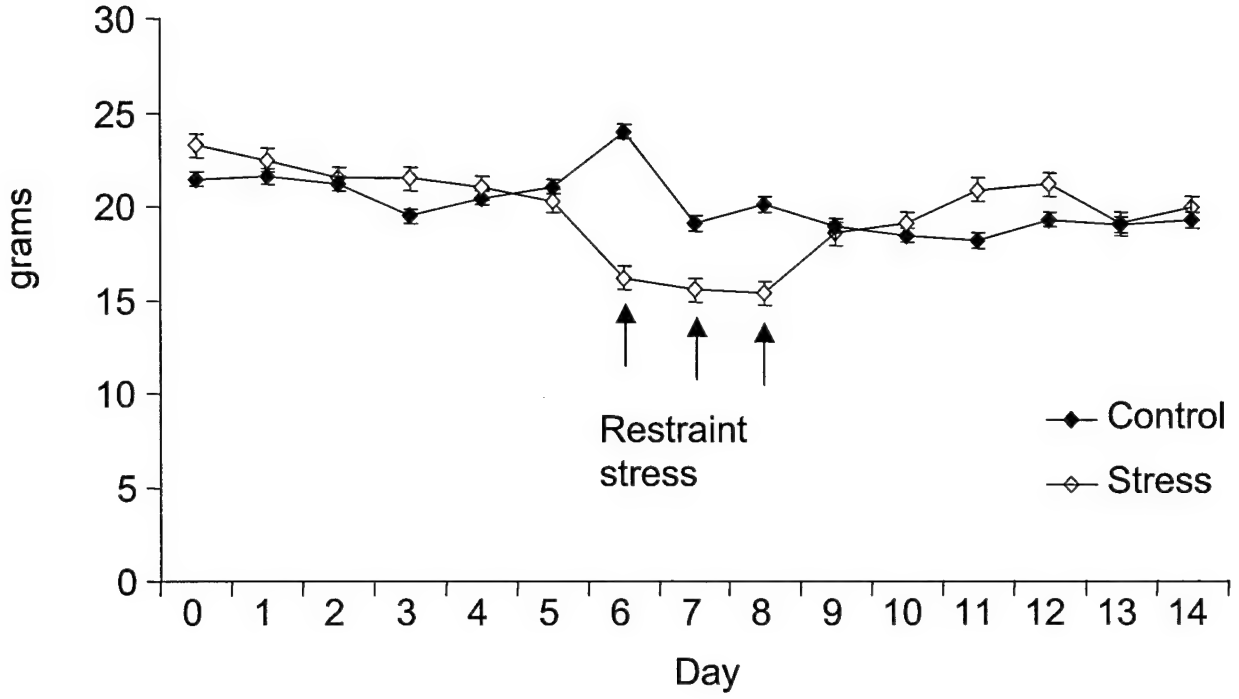


Figure 8: The effects of repeated restraint stress on feed intake of rats housed in calorimetry chambers

Data are means ± sem for groups of 6 rats

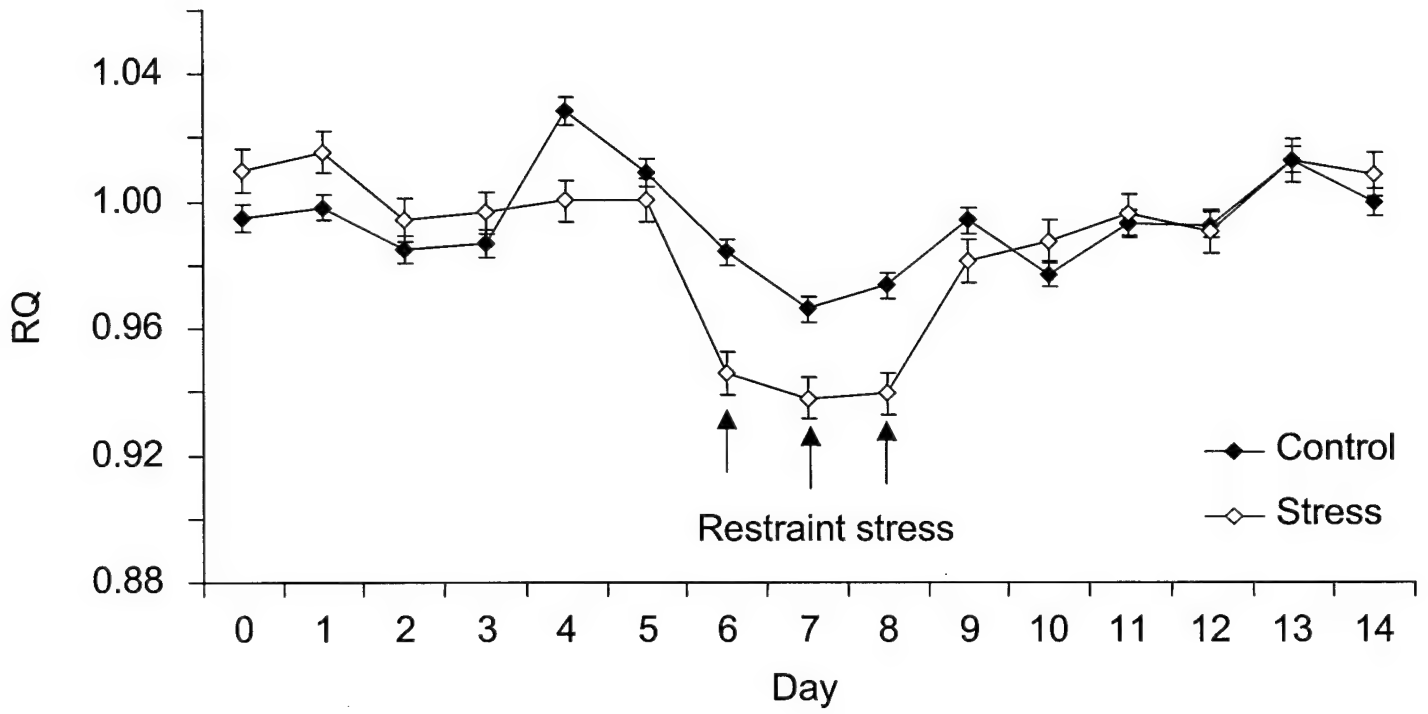


Figure 9: The effects of repeated restraint stress on respiratory quotient

Data are means ± sem for groups of 6 rats

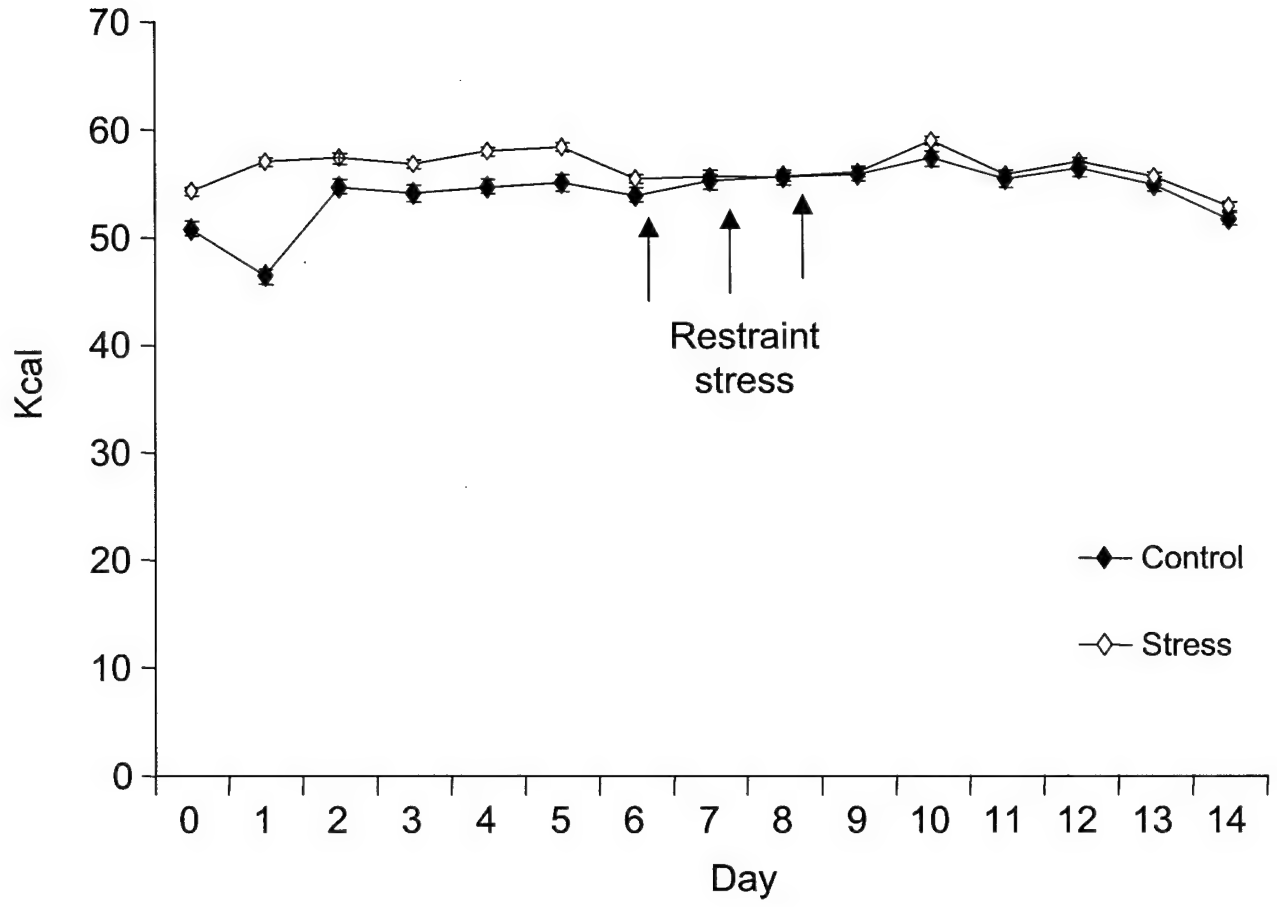


Figure 10: The effects of repeated restraint stress on heat production. Data are means ± sem for groups of 6 rats

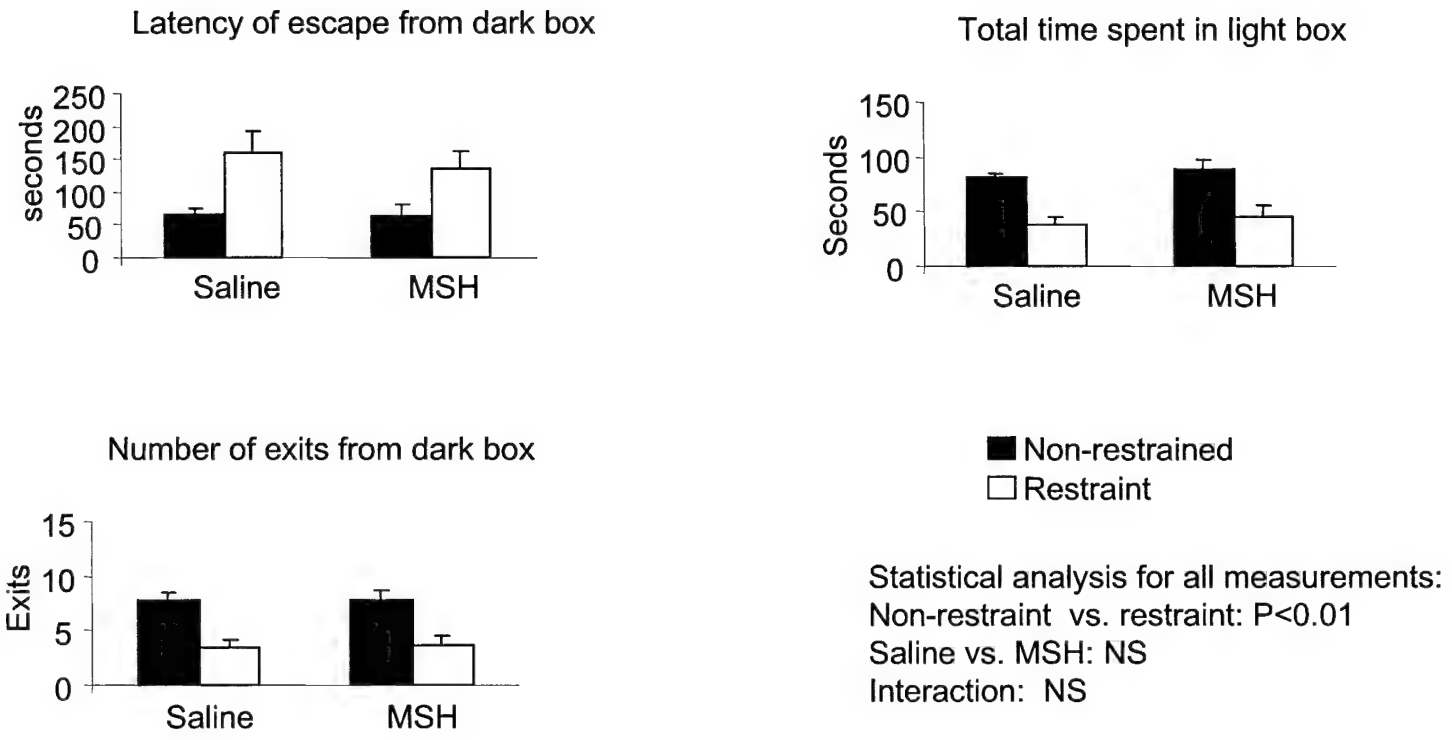


Figure 11: Anxiety behavior measured in the light dark box of mice pretreated with saline or αMSH prior to a 12 minute restraint stress. There was a significant effect of stress but no effect of αMSH on all three behavioral measures.

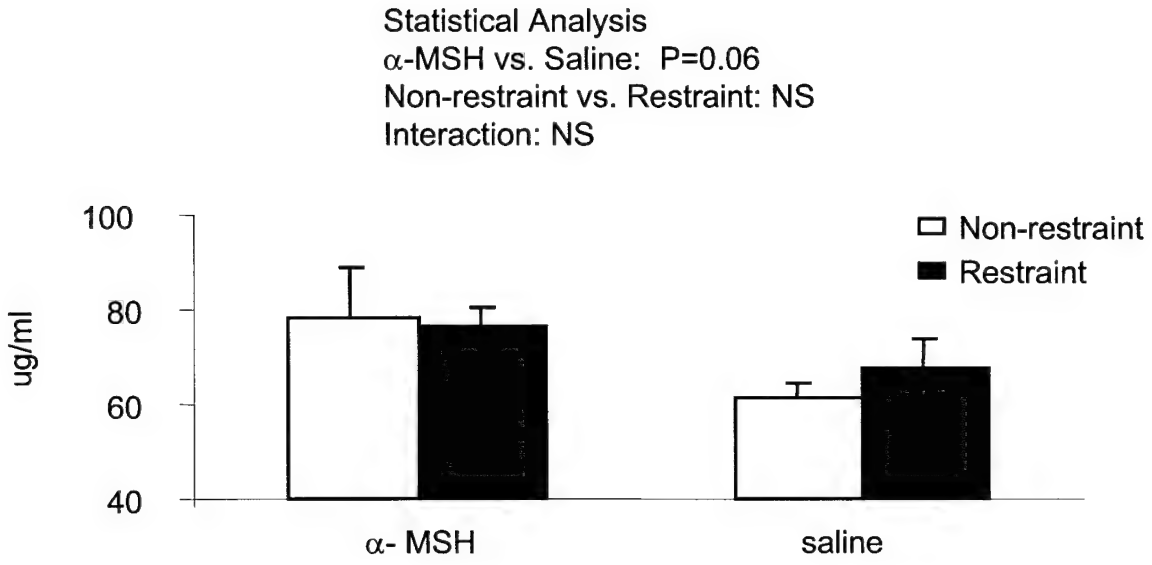


Figure 12: Plasma corticosterone. Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug αMSH 30 minutes before the start of restraint. Blood was collected by tail bleeding after 30 minutes of the first of 3 daily 2 hour periods of restraint.

Statistical analysis
α-MSH vs.. Saline: NS
Non-restraint vs.Restraint: P<0.01
Interaction: NS

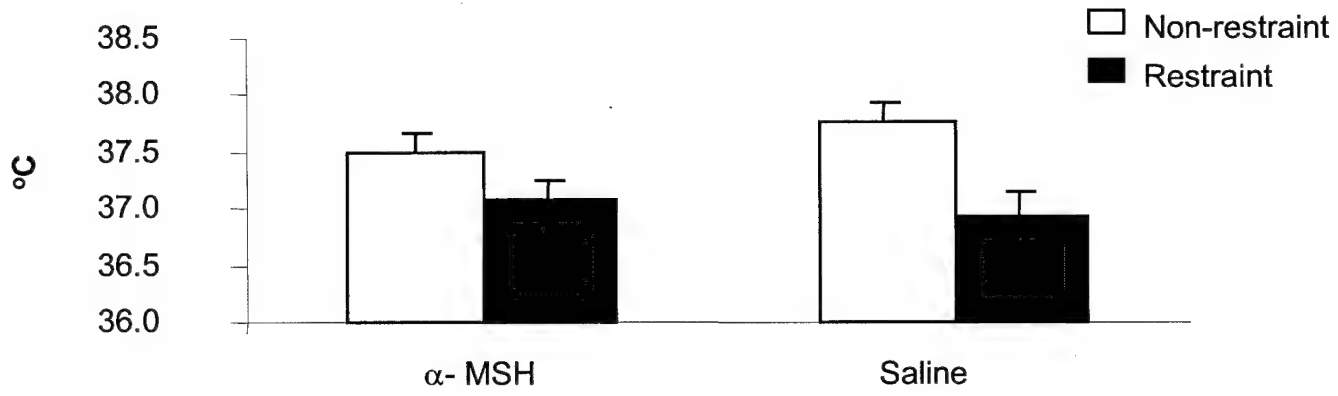


Figure 13: Rectal Temperature. Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug α-MSH 30 minutes before the start of a 2 hour restraint. Rectal temperature was measured at the end of the third of 3 daily periods of restraint.

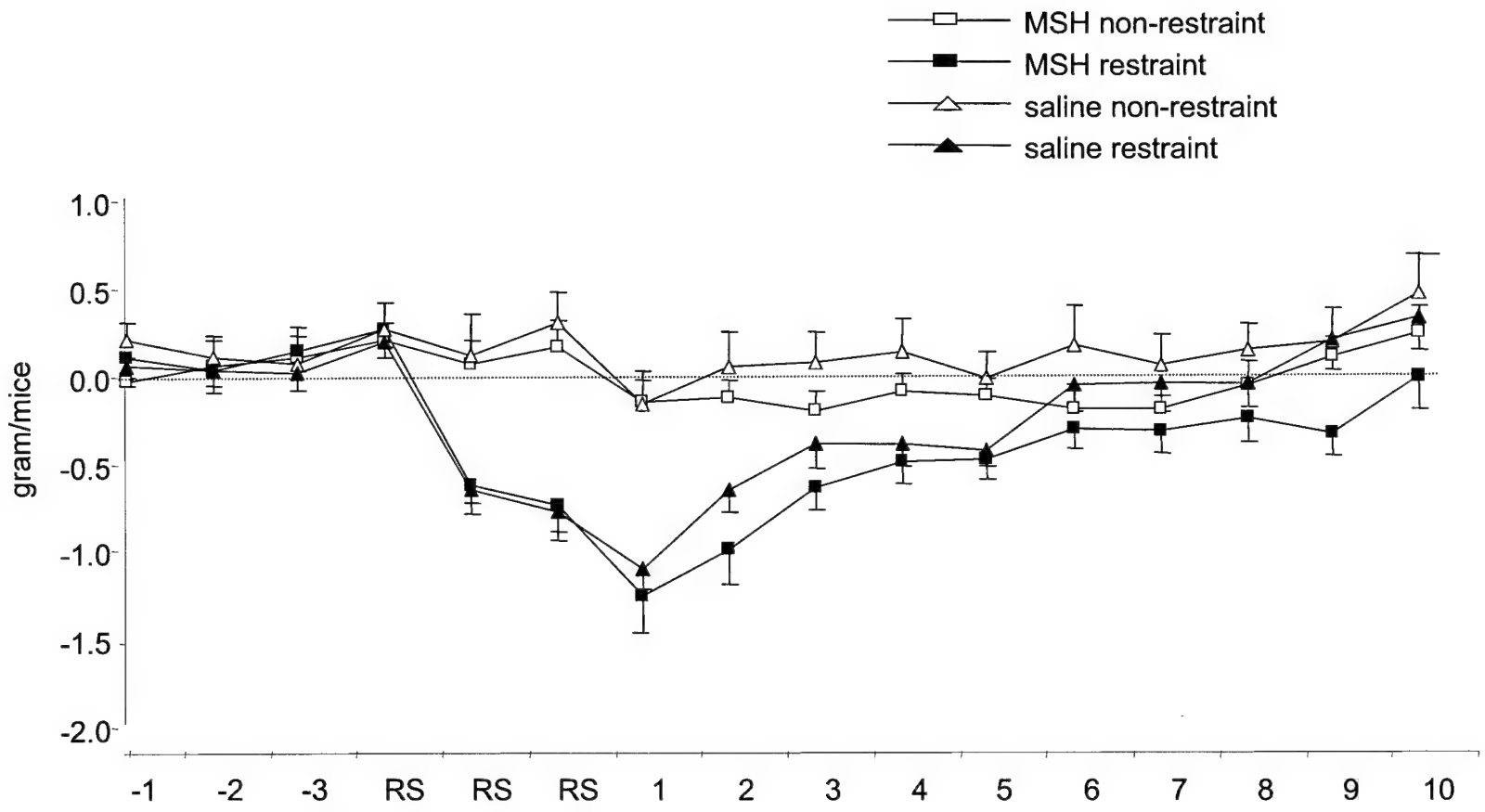


Figure 14: Body weight change in mice exposed to repeated restraint Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug αMSH 30 minutes before the start of a 2 hour restraint on each of the 3 days marked RS.

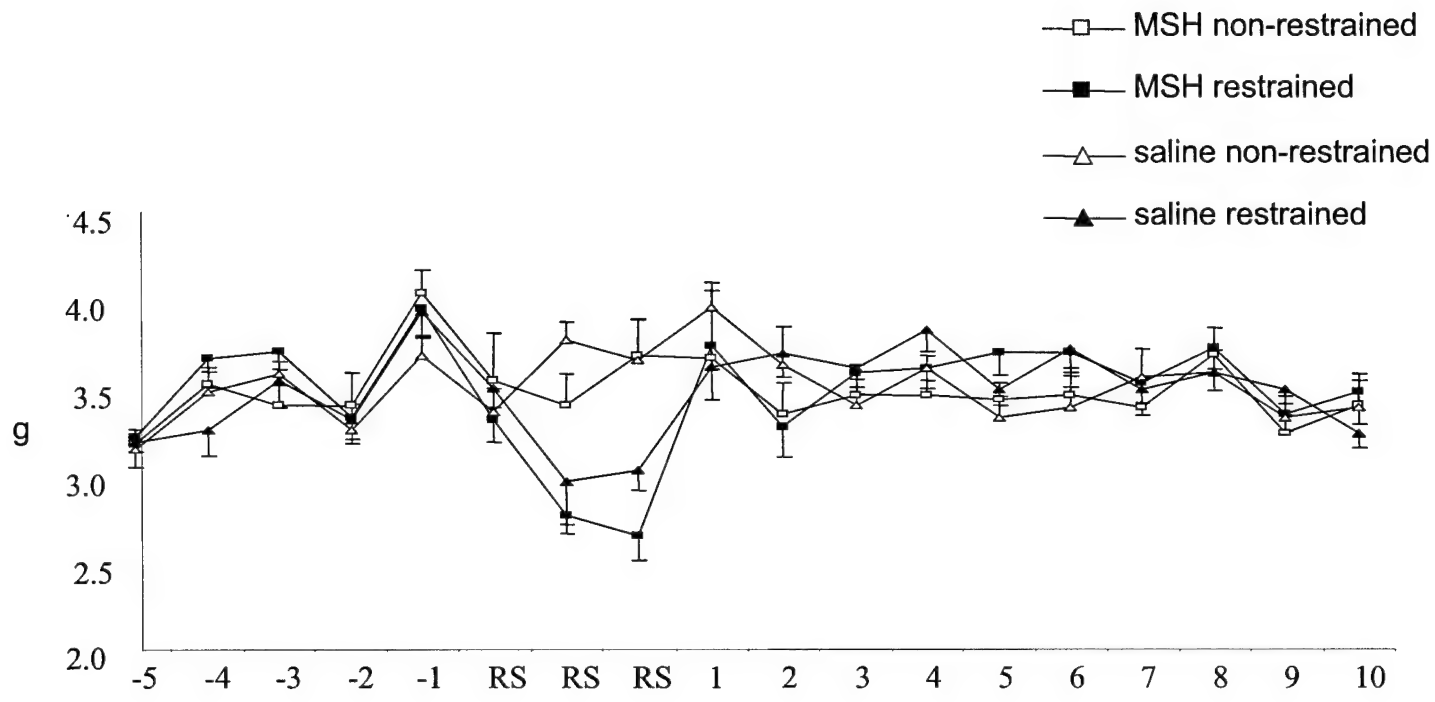
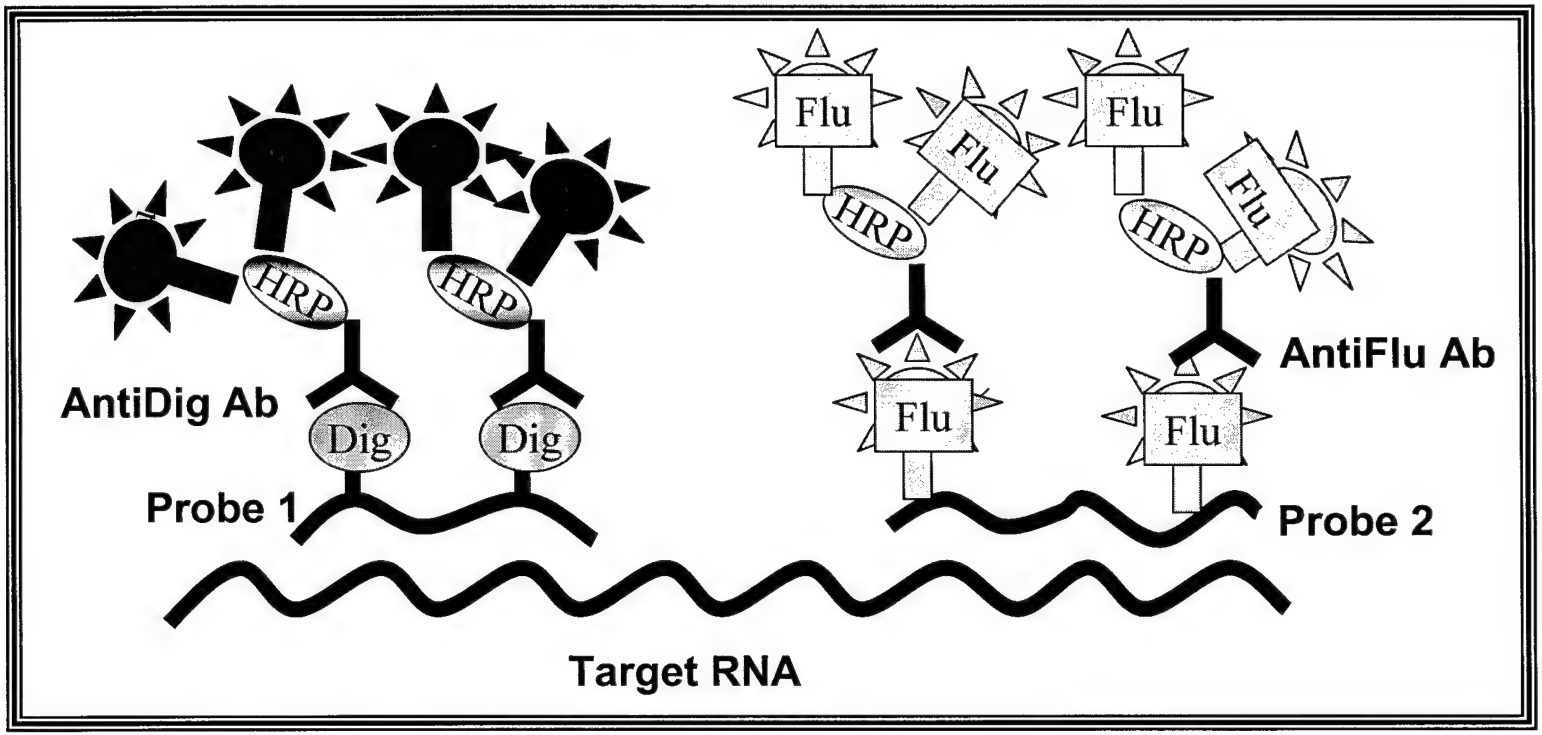
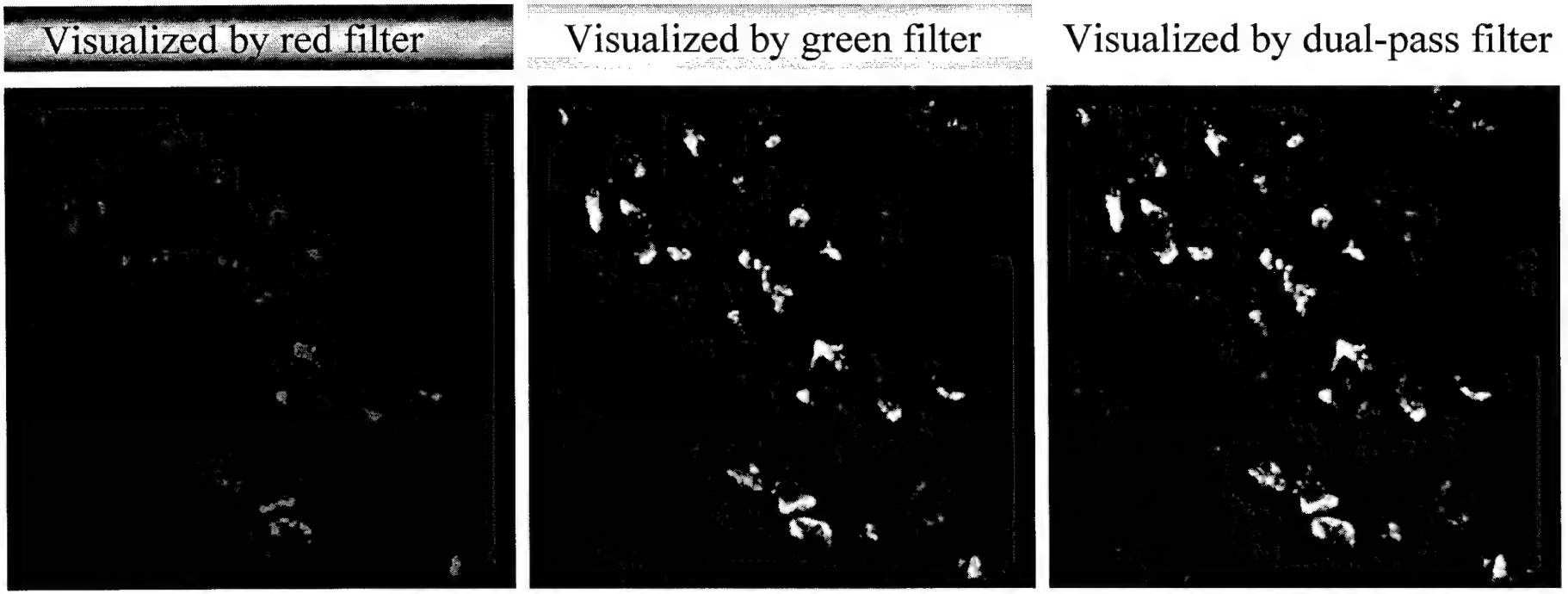


Figure 15: Food intakes of mice exposed to repeated restraint stress. Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug aMSH 30 minutes before the start of a 2 hour restraint on each of the 3 days marked RS.



Advantage:
1. High sensitivity: Amplified signal
2. Multiple target detection: If the two target gene express at the same cell, the yellow fluorescence should be visualized by using dual-pass filter

Figure 16. Carton illustration of principle for double color fluorescence in situ hybridization



Dopamine transport mRNA expression in substantia nigra. Photomicrographes were taken: (A) with red fluorescence filter to allow visualization of digoxigenin-labeled probe, (B) with green fluorescence filter to allow visualization of fluorecein-labeled probe, (C) with dual-pass filter to allow visualization of both probes.

Figure 17. Result of double color fluorescence in situ hybridization

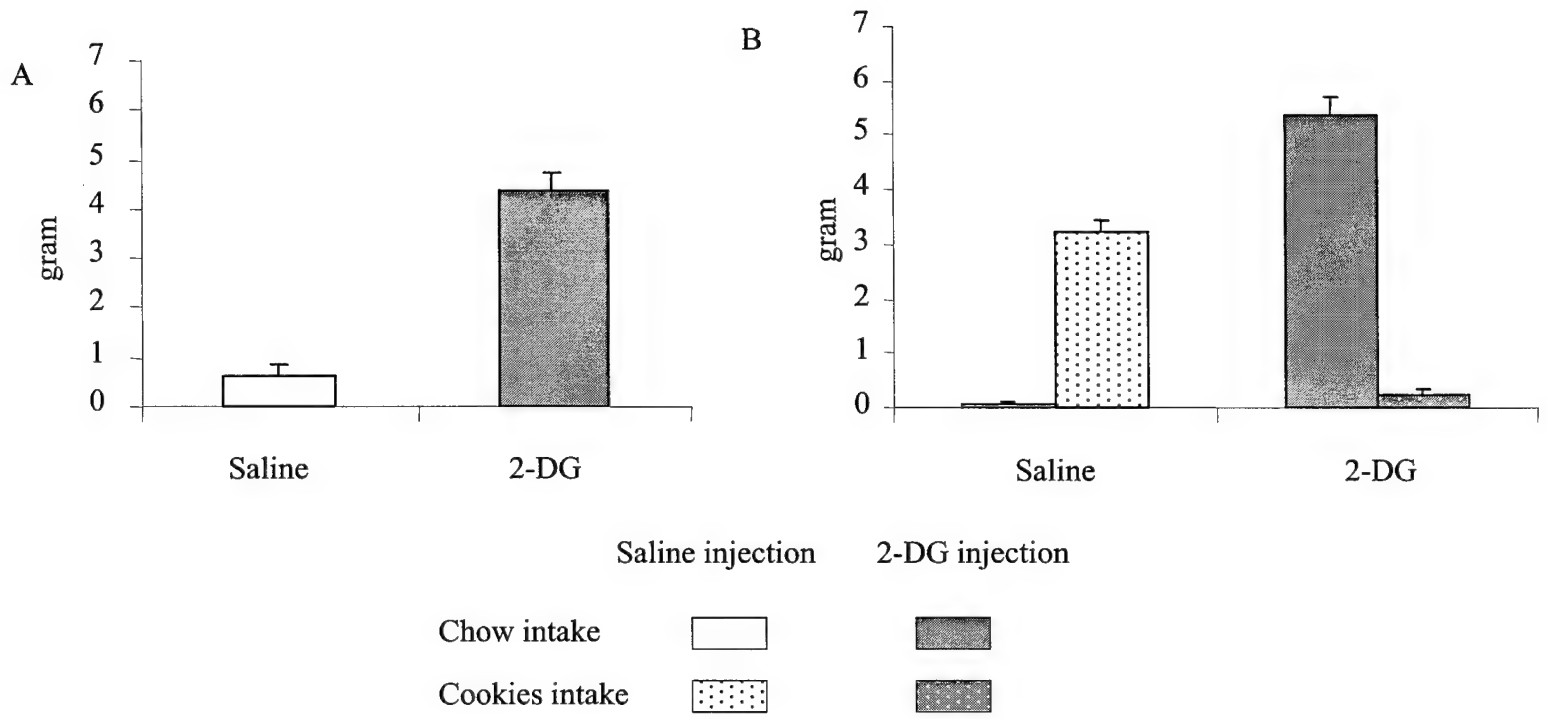


Figure18: Food intakes recorded four hours after injection saline or 2-DG. Data are means ± SE for group of 10 rats. Panel A shows chow intake in the rats fed chow only. 2-DG injection significantly increased chow intake compared with saline injected rats. ($P<0.01$). Panel B shows chow and cookies intake in the groups rats fed chow and cookies. Saline injection group only ate cookies and 2-DG injection rats only ate chow within four hours after injection.

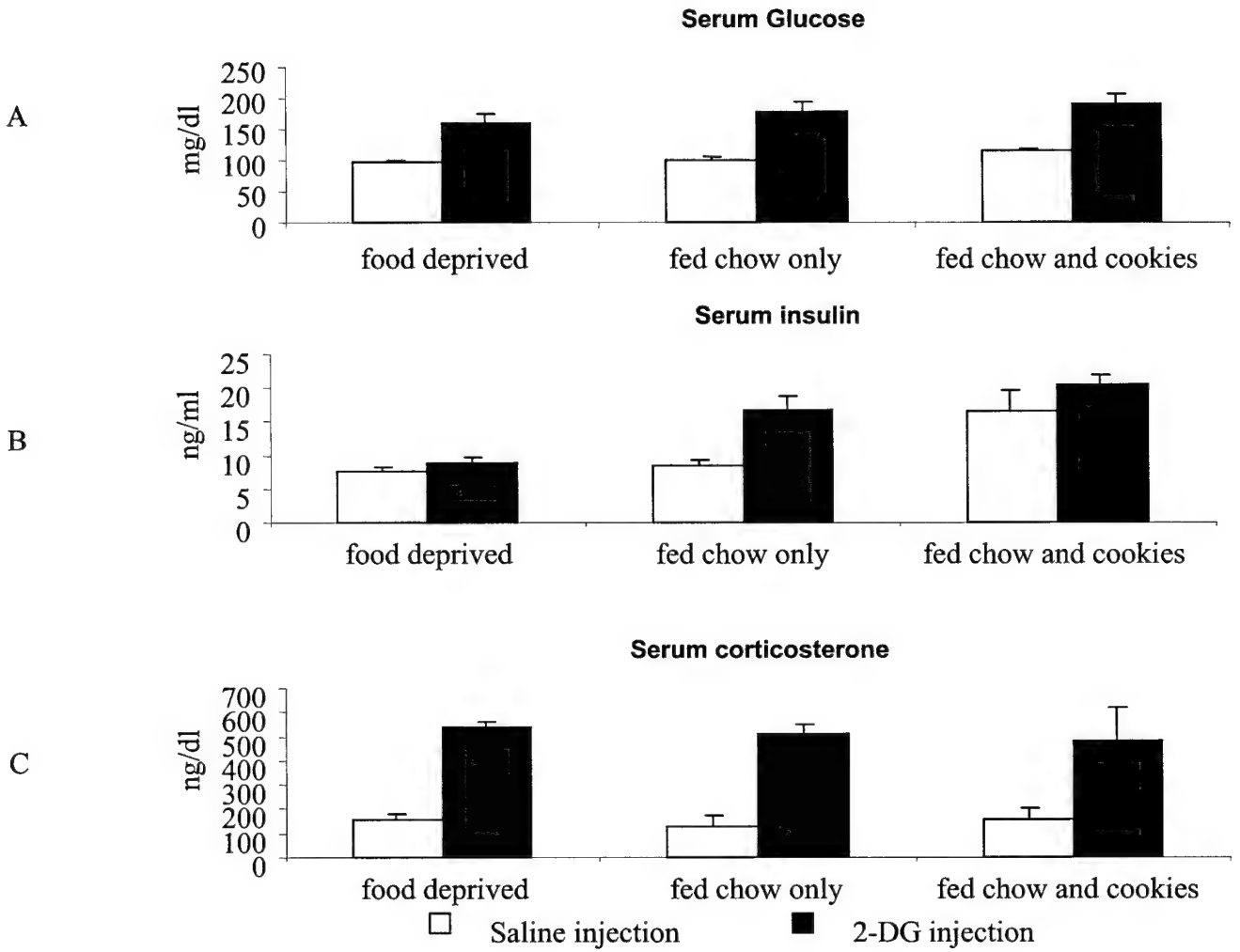


Figure 19: Serum assay results in rats injected with saline or 2-DG. Samples were collected at the end of four hours after injection and data are means ± SE for group of 4 or 5 rats. Panel A, B, and C show serum glucose, insulin, and corticosterone levels respectably. 2-DG injection significantly increased serum glucose and corticosterone levels in all three feeding regime groups compared with saline injection. Serum insulin level was only increase in fed chow only group rats after 2-DG injection.

Food deprived

Fed chow only

Fed chow and cookies

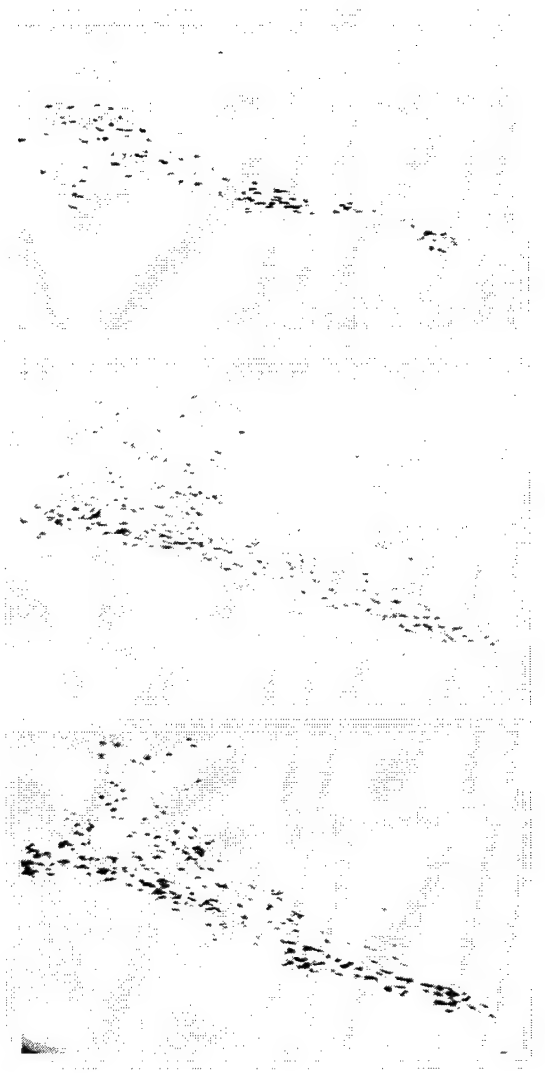


Figure20: Dopamine transport mRNA expressing in substantia nigra and ventral tegmentum measured by in situ hybridization in saline injection rats.

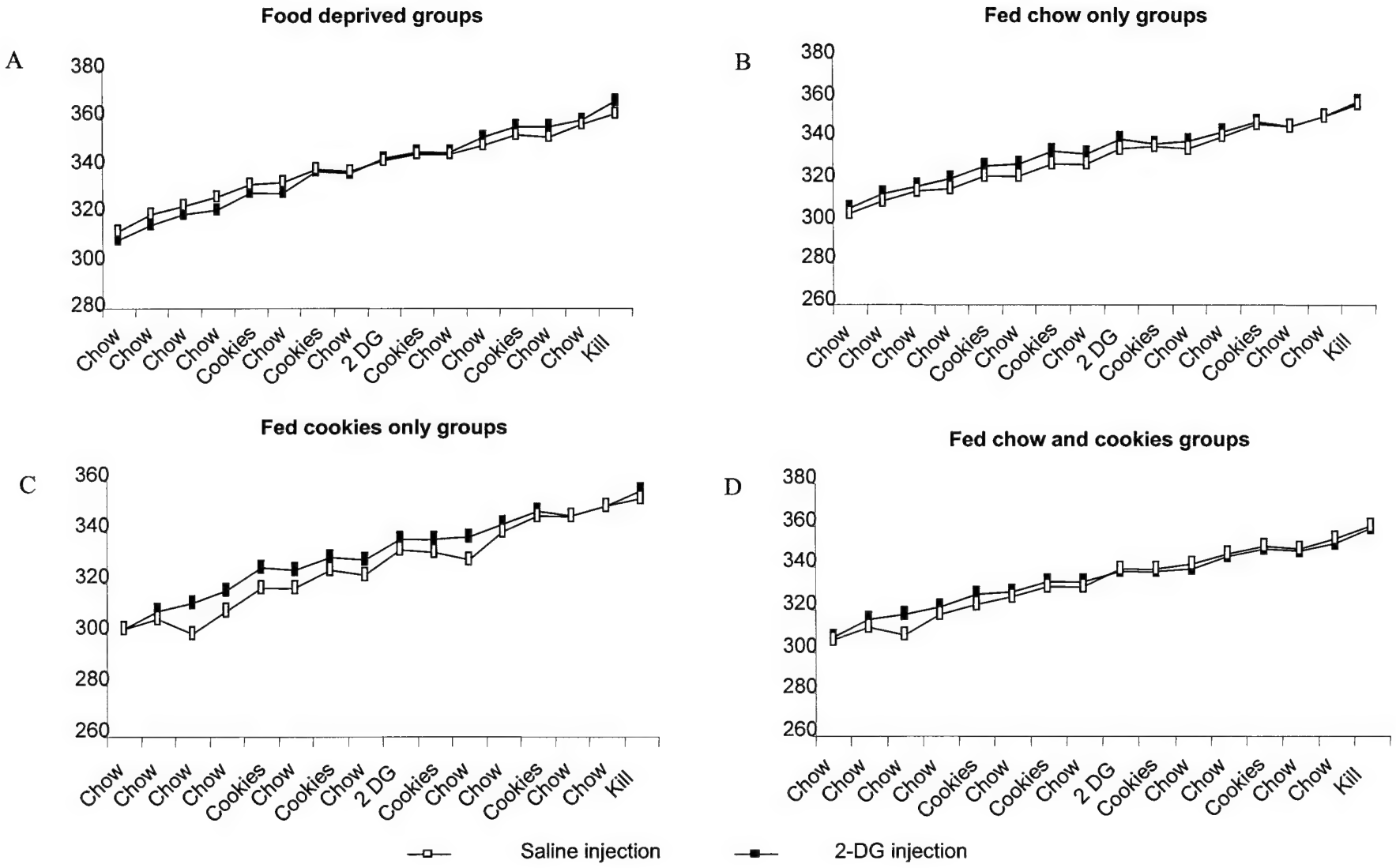


Figure 21: Body weight (grams) of food deprived (A), fed chow only (B), fed cookies only (C), and fed both chow and cookies (D) in saline and 2-DG injection rats. Data are mean of 7 or 8 rats. There is no difference in any of these groups compared saline with 2-DG injection.

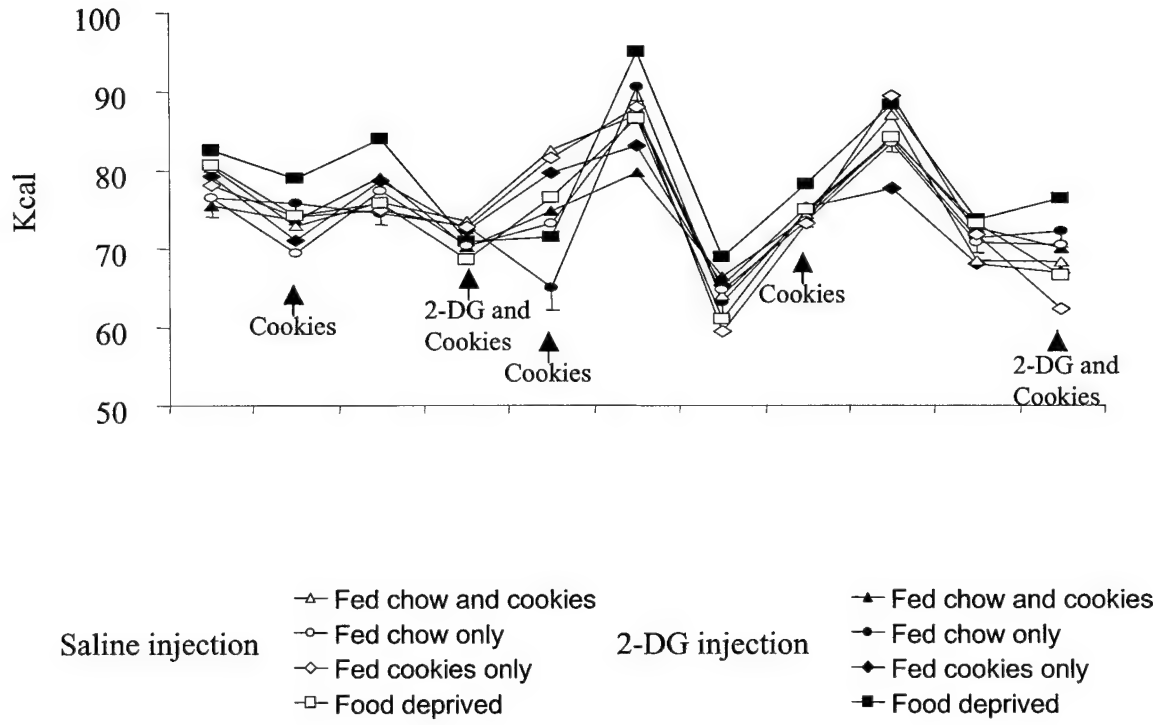
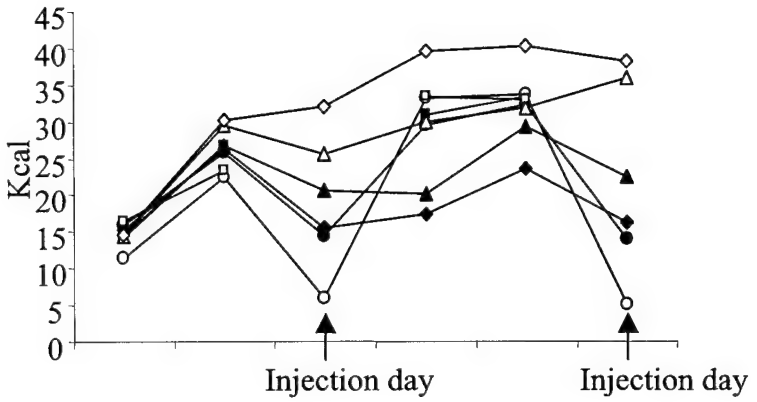
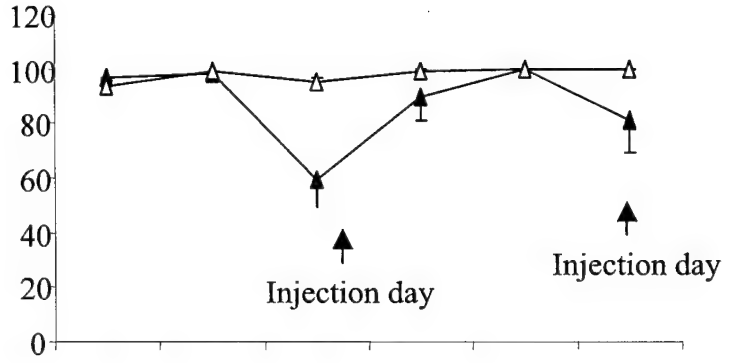


Figure22: Daily energy intakes of rats with multiple exposure to cookies and 2-DG injections. Data are means of 7 or 8 rats per group. Statistic analysis is in processing

A



B



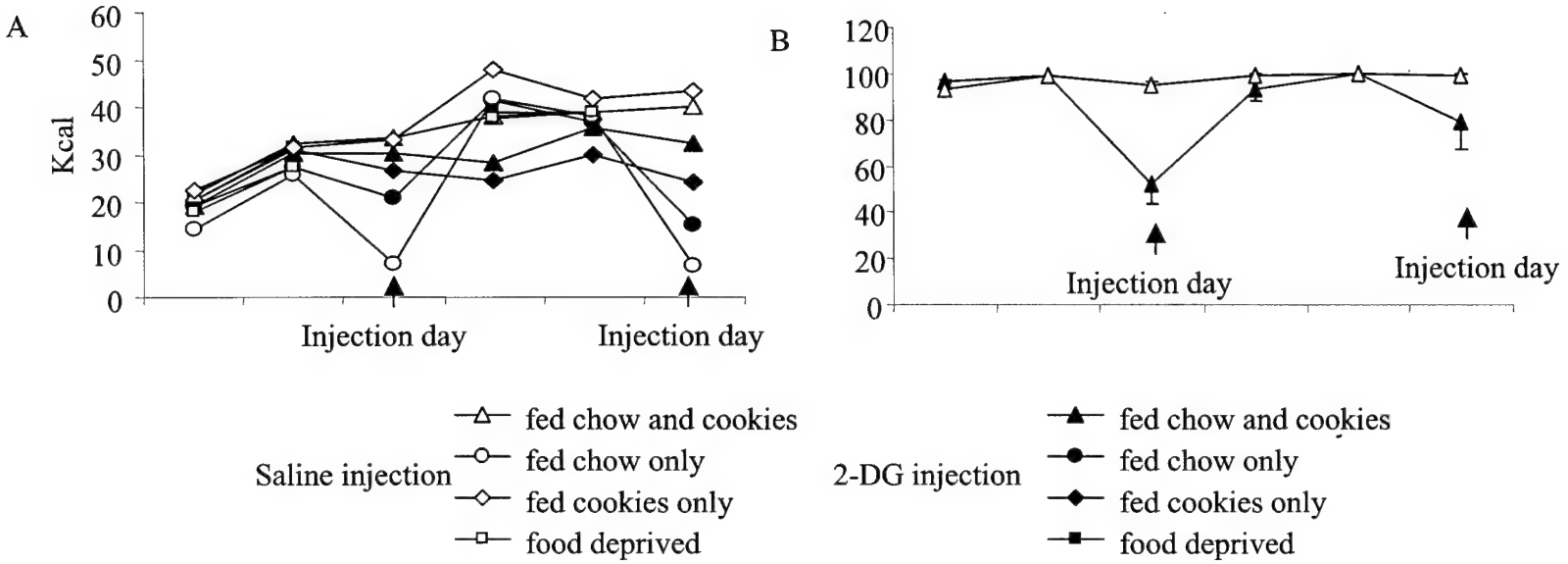
Saline injection
- fed chow and cookies
- fed chow only
- fed cookies only
- food deprived

2-DG injection
- fed chow and cookies
- fed chow only
- fed cookies only
- food deprived

C

	4 day before 1st injection	2 day before 1st injection	1st Injection	1 day after 1st injection	4 day after 1st injection	2nd injection
Control	7/7	7/7	7/7	7/7	7/7	7/7
2-DG	8/8	8/8	4/8	7/8	8/8	6/8

Figure 23: Energy intake and preference within first two hours after cookies was offered in rats with multiple exposure to cookies and 2-DG injections. Data are means of group 7 or 8 rats. Panel A: Energy intake. Panel B: % of energy come from cookies. Panel C: number of rats chose cookies over chow. Statistic analysis was in processing



C

	4 day before 1st injection	2 day before 1st injection	1st Injection	1 day after 1st injection	4 day after 1st injection	2nd injection
Control	7/7	7/7	7/7	7/7	7/7	7/7
2-DG	8/8	8/8	4/8	7/8	8/8	6/8

Figure24: Energy intake and preference within four hours after cookies was offered in rats with multiple exposure to cookies and 2-DG injections. Data are means of group 7 or 8 rats. Panel A: Energy intake. Panel B: % of energy come from cookies. Panel C: number of rats chose cookies over chow. Statistic analysis was in processing

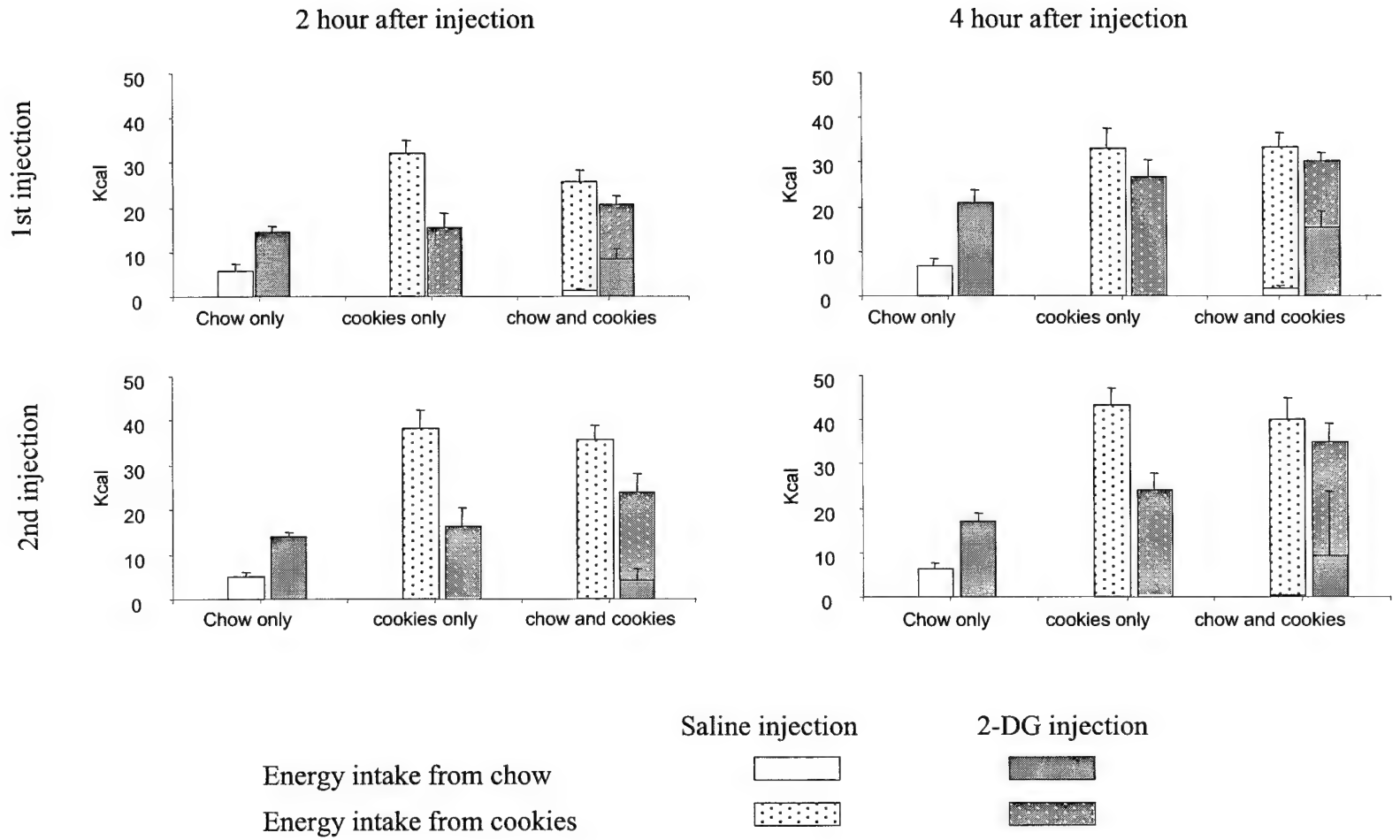


Figure 25: Energy intakes and preference within 2 and 4 hours after injection on injection day in rats with multiple exposure to cookies and 2-DG injections. Data are means± SE for 7 or 8 rats per group. Statistic analysis is in processing.

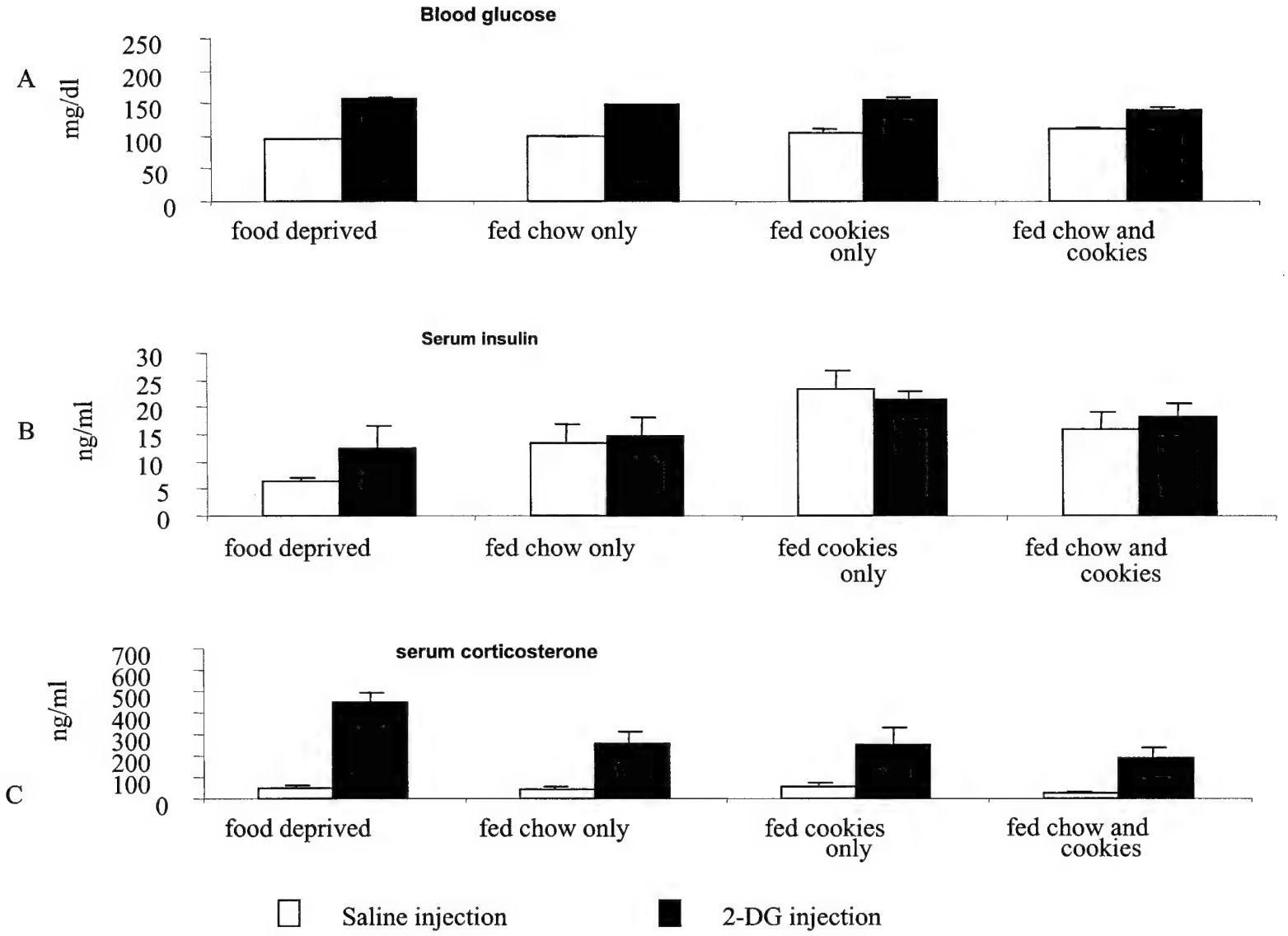
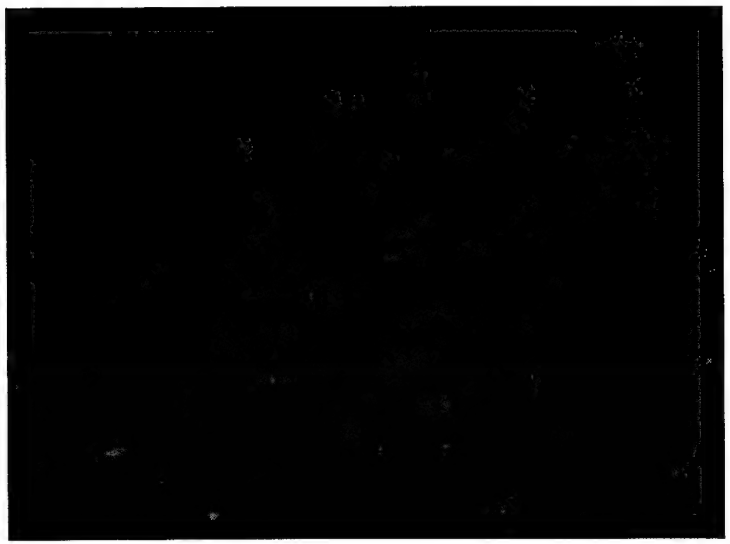
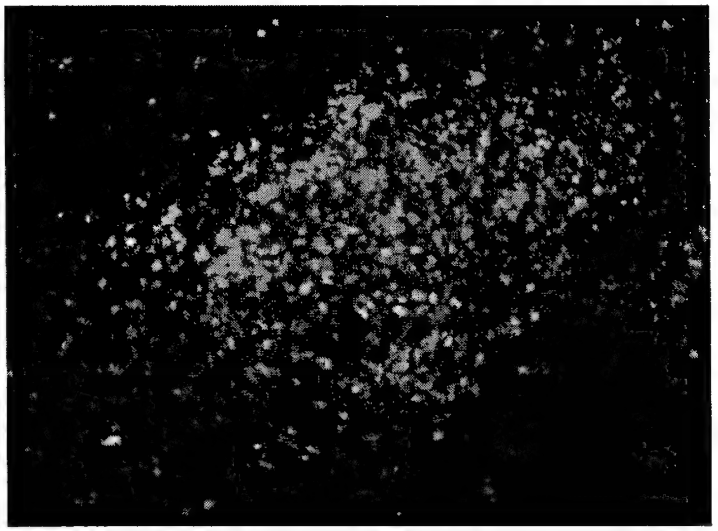


Figure26: Serum assay results in rats with multiple exposure to cookies and 2-DG injections. Samples were collected at the end of four hours after the last injection and data are means ± SE for group of 7 or 8 rats. Panel A, B, and C show serum glucose, insulin, and corticosterone levels respectably. Statistic analysis is in processing.



Glucokinase (GK)



GLUT2

Figure 27: Co-expression of GK and GLUT2 mRNA at mlf in hindbrain (Bregma -14.00mm) measured by double-labeled fluorescence in situ hybridization

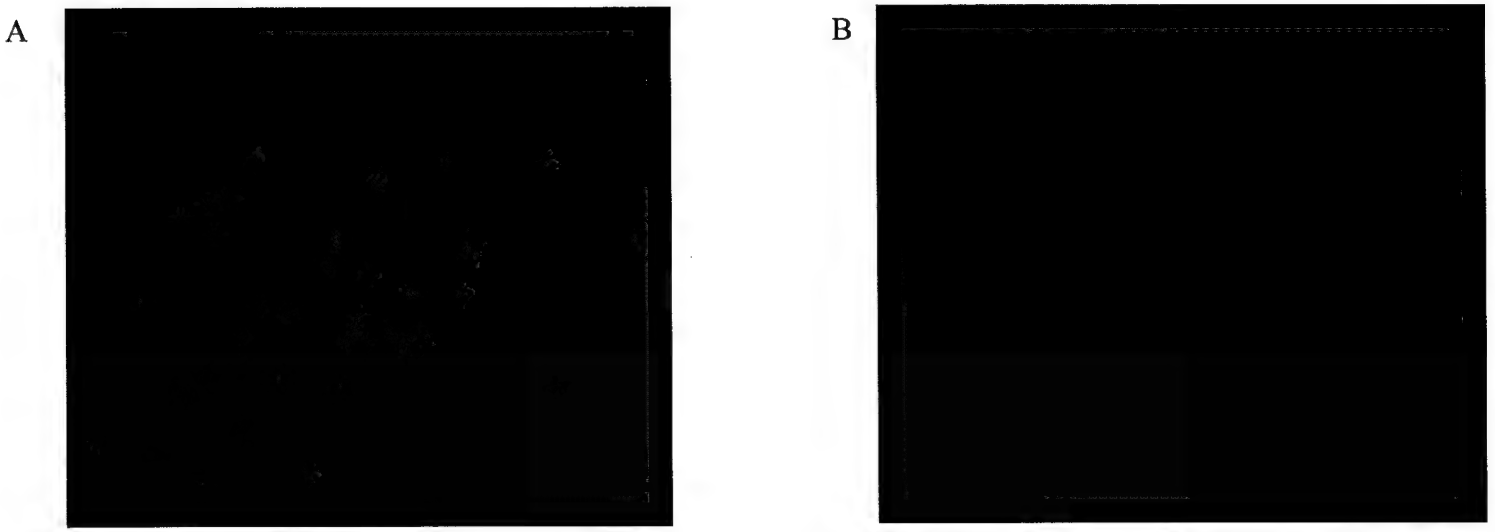


Figure 28: Glucokinase (GK) expressed at mlf of hindbrain measured by double-labeled fluorescence in situ hybridization. A: 200X amplification B: 100x amplification

TASK IV

STRESS, NUTRITION AND WORK PERFORMANCE

Short-Term Dietary Energy Restriction Reduces Lean Body Mass but Not Performance in Physically Active Men and Women

J. J. Zachwieja, D. M. Ezell, A. D. Cline, J. C. Ricketts, P. C. Vicknair, S. M. Schorle, D. H. Ryan

Exercise and Nutrition Program, Pennington Biomedical Research Center, Baton Rouge, LA, USA

Zachwieja JJ, Ezell DM, Cline AD, Ricketts JC, Vicknair PC, Schorle SM, Ryan DH. Short-Term Dietary Energy Restriction Reduces Lean Body Mass but Not Performance in Physically Active Men and Women. Int J Sports Med 2001; 22: 310–316

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We studied the effect of moderate, short-term energy restriction on physical performance in physically fit men (n = 13) and women (n = 11) in a controlled clinical research setting with a metabolic kitchen, exercise testing laboratory and training facility. The experiment consisted of a 10 d baseline period followed by either 2 wk of dietary energy restriction (750 kcal/d; n = 16) or energy balance (control; n = 8). During this 24 day study, exercise energy expenditure averaged 465 ± 5.7 kcal/d in all subjects and was accomplished through treadmill running at a self-selected pace. Body weight was maintained in the control group (– 0.36 ± 0.24 kg), but energy restriction resulted in weight loss of – 1.29 ± 0.16 kg ($p < 0.001$). There was a trend for lean body mass to decline more in the energy restriction group ($p = 0.093$), accounting for 61 % of the weight loss, and urinary nitrogen excretion also tended to be higher in the energy restriction vs. control group (i.e., 13.2 ± 1.1 vs. 11.2 ± 1.0 g/d; $p = 0.089$). Muscle strength (leg & shoulder press; 1 repetition maximum) was maintained or increased during the energy restriction period. Muscle endurance, assessed by leg squats to fatigue, and 5 mile run time improved following two weeks of energy restriction or balance. Anaerobic capacity (Wingate Test) increased slightly in the restriction (+ 368 ± 219 joules) but declined in the control group 649 ± 288 joules; $p<0.05$). We conclude that short-term (2 weeks) moderate energy restriction (~750 kcal/d) results in weight loss but does not impair performance in physically fit young men and women.

■ Key words: Weight loss, body composition, diet, athletes, aerobic and anaerobic performance.

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Introduction

A number of studies have been conducted to evaluate the effect of weight loss on physical performance [5,8,11,12]. This has been important because weight loss may be required to achieve weight standards for a particular sport (i.e., gymnastics), to adhere to weight classifications for different sporting events (i.e., wrestling), or to achieve a goal weight for a particular team sport position. In most cases, hypoenergy diets have been used to induce weight loss; however, it is now recognized that rapid weight loss accomplished through hypoenergy dieting with fluid restriction can result in significant decrements in anaerobic exercise performance, particularly when the diet is low in carbohydrate [8,11].

Excessive physical activity or training can result in unintentional weight loss. Generally, the weight loss is of slower onset and likely arises from an uncoupling of *ad libitum* energy intake and expenditure [9]. For example, rigorous military field training is known to induce energy deficits as high as 1000 kcal/d resulting in a sizable weight loss [6,10]. Understanding the shortfall in *ad libitum* energy intake and weight loss in this condition is complicated by the coexistence of other operational stressors such as sleep deprivation and climatic extremes. Still, in other settings, the so-called "anorectic" effect of exercise is present. Swim training at a volume of 2× normal was associated with an energy intake that did not fully compensate for increased expenditure [1]. Further, some swimmers encounter chronic muscle fatigue during a sudden increase in training volume and this relates to failure to ingest sufficient carbohydrate [4]. Irrespective of weight loss, insufficient energy intake has the potential to negatively impact aerobic as well as anaerobic exercise performance. Few detailed and carefully controlled studies have been conducted to test this hypothesis. Therefore, the purpose of this investigation was to determine the effect of two weeks of moderate energy restriction (750 kcal/d) on exercise performance in physically active men and women.

Methods

Subjects

Thirteen men and 11 women who regularly performed aerobic exercise were recruited to participate in this study. All were healthy as determined by medical history and physical exami-

nation, which included routine blood and urine chemistries. None had experienced significant weight loss/gain (> 5 kg) in the previous 6 months, nor were they routinely consuming dietary supplements such as creatine monohydrate. All had a habitual dietary protein intake of between 0.8 and 2.0 g/kg/d. Physical characteristics of the subjects are given in Table **1**. The Pennington Biomedical Research Center and Department of Defense Institutional Review Boards approved this study and all subjects gave written informed consent.

Table 1 Descriptive characteristics of the subjects

	Age (yrs)	Weight (kg)	% Body Fat (DEXA)	BMI	$\dot{V}O_2$max (ml/kg/min)
Total (n = 24)	24 ± 4	71.7 ± 11.9	22.9 ± 7.8	24.1 ± 2.8	51.1 ± 5.0
Men (n = 13)	25 ± 4	80.1 ± 8.5	19.3 ± 7.2	25.3 ± 2.8	53.4 ± 4.7
Women (n = 11)	21 ± 3	61.8 ± 6.6	27.6 ± 6.1	22.7 ± 2.2	48.8 ± 4.4

Data are mean ± SD. Self-reported habitual physical activity: the primary mode of physical activity for these subjects was running (21/24). 19% ran 1 – 2× per week, 52% ran 3 – 4× per week and 29% ran 5 – 6× per week. A typical running session was reported to last 30 – 40 minutes.

Experimental design

The study consisted of 24 days of physical training in the laboratory with pre- and post-intervention measurements of body composition, muscular strength and endurance, running performance, anaerobic capacity, and urinary nitrogen excretion (Fig. **1**). Prior to experimental testing, basal metabolic rate was determined by indirect calorimetry and maximal oxygen consumption (max $\dot{V}O_2$) was measured during an incremental test to exhaustion while running on a treadmill. Before the start of the study, subjects completed a three-day food record, which was used to determine habitual energy and protein intake. In order to decrease any potential experimental artifact related to individual differences in energy balance and diet composition during the baseline period (days 1 – 10), and to ensure controlled energy intake during the intervention period (days 11 – 24), subjects were fed diets designed and prepared by the Pennington Center metabolic kitchen.

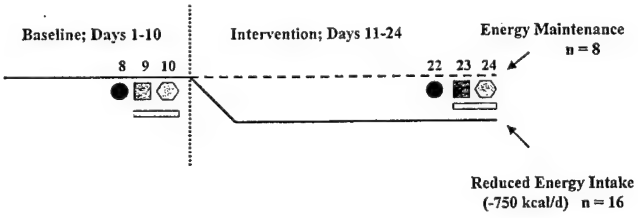

Fig. 1 Schematic of study design. All subjects participated in a 10-day baseline period and were then randomized to consume a reduced calorie diet or to continue on an energy maintenance diet for 14 days. Daily treadmill exercise, sufficient to expend 500 kcal, was performed throughout the 24 day study. Measurements of exercise performance, body composition, and urinary nitrogen excretion were made at the end of the baseline and intervention periods. Circle; muscle endurance Test, square; muscle strength and Anaerobic Power Test, hexagon; Body composition and 5-mile run Test, unfilled bar; urine collection for Nitrogen Excretion.

Baseline period (study days 1 – 10)

Subjects reported to the metabolic kitchen for breakfast and dinner meals. Snacks and lunch were provided for off-campus consumption. Daily energy intake was adjusted for each subject's individual energy needs to prevent weight loss or gain. In addition, subjects reported daily to the laboratory to perform treadmill exercise predetermined to elicit an energy expenditure of ~500 kcal.

Baseline measurements

On day eight of the baseline period, subjects completed a muscle endurance test. On day nine, subjects completed tests for muscular strength and anaerobic performance. These tests were performed before or at least four hours after treadmill exercise. On day ten, lean body mass and percent body fat were determined by dual energy X-ray absorptiometry (DEXA). In place of that day's treadmill exercise bout, subjects participated in a 5-mile running performance test on an indoor track. On days nine and ten all urine voids were collected for an assessment of 24 h urinary nitrogen excretion.

Intervention period (study days 11 – 24)

Subjects continued daily treadmill exercise, each bout sufficient to elicit an energy expenditure of ~ 500 kcal. Eight subjects (5 men and 3 women) were randomly assigned to continue a diet, which met energy needs and maintained body weight, while 16 subjects (8 men and 8 women) consumed diets that reduced daily energy intake by 750 kcal/d. All subjects and primary investigators were blinded to dietary treatment during this intervention period. On day 22, subjects repeated the muscle endurance test. On day 23, tests of muscle strength and anaerobic performance were repeated. On day 24, body composition by DEXA was re-assessed, and in place of the final treadmill exercise bout, subjects repeated the 5-mile running performance test. On days 23 and 24 all urine voids were collected as described above for a reassessment of 24 h urinary nitrogen excretion.

Diets

Energy intake during the baseline period was designed to meet each subject's individual energy needs as determined from a basal metabolic rate measurement and from 3-day dietary records obtained during screening. Adjustments were made to account for the 500 kcal/d treadmill exercise energy expenditure. During the intervention period, a majority of the 750 kcal/d energy deficiency was achieved by withholding carbohydrate (~ 60% of the deficit), while protein intake was held constant. A comparison of the macronutrient composition of the baseline and intervention diets can be found in Table **2**. Diets were designed to include standard foods consumed in a regular American diet, meeting the nutritional recommendations of the US Recommended Dietary Allowances. One standard menu cycle (5 days) was designed for all study participants. Subjects were interviewed for individual food allergies or intolerances, and food substitutions were made if necessary. Body weight was determined each morning prior to breakfast. Subjects were weighed on a calibrated, electronic scale in "street clothes" after removing shoes and wallets, coins, keys etc. from pockets. During the intervention period, subjects were not al-

Table 2 Comparison of the macronutrient content of the control and energy restrictive diets

	Baseline/Control Diet			Energy Restriction Diet		
	Percent kcal	Grams	Grams/kg	Percent kcal	Grams	Grams/kg
Carbohydrate	51%	383	5.2	47%	264	3.7
Fat	35%	117	1.6	34%	84	1.2
Protein	14%	105	1.4	19%	105	1.4

These values are based on a 3000 kcal baseline/control diet and a 2250 kcal energy restrictive diet. Actual percentages and gram intakes varied according to individual needs. Relative intake of macronutrients is based on a body weight of 72 kg.

lowed to view their body weight and were asked not to weigh themselves at home or another location.

Experimental measurements

Maximal oxygen consumption (max $\dot{V}O_2$)

Each subject was tested for max $\dot{V}O_2$ on a treadmill using an incremental work protocol. After establishing a sustainable pace, the treadmill incline was increased by 2% every minute until exhaustion while $\dot{V}O_2$ and $\dot{V}CO_2$ were continuously monitored with a SensorMedics Metabolic Cart (Vmax Series 29). Max $\dot{V}O_2$ was defined as the highest 20 second average $\dot{V}O_2$ achieved during the last 90 seconds of the test. Attainment of max $\dot{V}O_2$ was accepted if two of the following three criteria were met: plateau in $\dot{V}O_2$ with increasing workload, maximum heart rate within 10 beats of the age predicted maximum heart rate, and expiratory exchange ratio greater than 1.1. Max $\dot{V}O_2$ was used to evaluate pre-trial fitness level of the subjects. To calculate average energy expenditure during running, after the subjects had a chance to recover from the max $\dot{V}O_2$ test, they ran on the treadmill at a self-determined "comfortable" pace for 1 mile. Near the end of the run, expired air was collected and analyzed using the SensorMedics metabolic cart. Energy expenditure per mile was calculated and used to determine the duration of treadmill exercise (at the set pace) for the baseline and intervention periods.

Body composition

Total body fat and lean body mass (LBM) were measured by DEXA. The instrument used was a Hologic QDR 2000, operated with the Enhanced Array Whole Body Software Package, version 5.678A. DEXA determinations were made in the morning on days 10 and 24 of the experiment.

Muscle endurance test

On days eight and 22, a lower body muscular endurance test was completed. This test consisted of squatting exercise with a 45.5 kg barbell weight for men and a 22.7 kg barbell weight for women. The subjects performed 25 repetitions per minute, timed with a metronome, and continued until exhaustion. Exhaustion was self-determined or defined as the point at which the subject could no longer keep up with the metronome. Total number of repetitions for the test was recorded.

Muscle strength test

On days nine and 23, a one repetition maximum (1 RM) protocol was used to determine upper body (UB) and lower body (LB) muscular strength using *BodyMasters™* resistance exercise equipment. The Shoulder Press machine was used to determine UB strength and the Super Leg Press was used to determine LB strength. Standardized instructions explaining each test and a demonstration of proper technique was given prior to testing. After a standardized warm-up of 15 repetitions at a manageable resistance, a weight close to, but under the subject's expected maximum lifting capacity was selected. If one repetition was completed, 4.5 kg increments were added to the exercise device until 1 RM was achieved.

Anaerobic capacity test

Following the muscle strength test on days nine and 23, a Wingate anaerobic capacity test was performed [2]. The test consisted of an all out 30 s maximal effort on a variable load Cybex™ cycle ergometer at a workload equal to 75 g/kg body weight. This workload was not adjusted for weight loss during energy restriction. Total work (in joules) performed during the 30 s test was calculated and used as a measure of anaerobic capacity.

Five-mile performance run

In place of the treadmill exercise session on days 10 and 24, subjects performed a timed 5-mile running test designed to measure endurance performance. Subjects reported to the indoor track at Louisiana State University's: Student Recreation Center at assigned times. Instructions and information such as number of laps per mile and proper lane usage followed a warm-up consisting of a 1-lap jog and standardized hamstring, quadriceps, groin and calf muscle stretches. Running performance was defined as each subject's time to complete 5 miles.

Urine collection and analysis

On days 9, 10, 23 and 24 subjects collected all urine voids. The urine specimens were divided into two 24 h periods, the total volume measured, recorded and an aliquot saved for analysis of total nitrogen content. Completeness of urine collection was determined by urinary creatinine measurements. Urinary nitrogen was measured by chemiluminescence using a model 703C pyrochemiluminescent system (Antek Instruments Inc., Houston, TX).

Statistical analysis

Body composition, urinary nitrogen excretion and performance results are presented as mean ± SE. Change from baseline was calculated for each of these variables and statistical differences determined by an unpaired t-test. To establish main effects, performance measures were also analyzed by repeated measures ANOVA. Body weight data are mean ± SE and were analyzed by repeated measures using a mixed model with an unstructured covariance matrix. Statistical significance was set at $p < 0.05$.

Results

Energy intake and exercise energy expenditure

Energy intake in the control group was constant throughout the study averaging ~ 3200 kcal/d (43.8 kcal/kg/d). As planned the restriction group consumed, on average, 725 fewer kcal/d during the intervention period as compared to baseline ($p < 0.0001$). The percent reduction in caloric intake was 24.1 ± 1.4% and ranged form 16.5% to 32.6%. Treadmill exercise energy expenditure averaged 470 ± 6 kcal/d during the baseline period and remained constant throughout intervention in both the control and restriction groups. Energy intake and exercise energy expenditure data can be found in Table **3**.

Table 3 Energy intake and exercise expenditure during the baseline and intervention periods

	Baseline	Intervention; days 11 – 17		Intervention; days 18 – 24	
		Control	Restriction	Control	Restriction
Energy intake					
(kcal/day)	3151 ± 118	3248 ± 198	2398 ± 155*	3247 ± 199	2400 ± 155*
(kcal/kg/d)	44.1 ± 1.6	44.7 ± 2.7	34.3 ± 2.2*	44.7 ± 2.7	34.4 ± 2.2*
Exercise energy expenditure					
(kcal/day)	470 ± 6	482 ± 8	463 ± 6	460 ± 8	459 ± 9

Data are mean ± SE. Weekly averages for energy intake and exercise energy expenditure were calculated for each subject during the baseline (days 1 – 10) and intervention periods. Change from baseline significantly different compared to control; *$p < 0.0001$

Body weight change

During the baseline period, body weight was stable in all subjects averaging 71.7 ± 2.4kg. There was a significant treatment × time interaction for body weight change during the intervention period ($p < 0.001$). As can be seen in Fig. **2**, the energy restriction group lost approximately 1.2 kg while weight loss for the control group was negligible. The percent reduction in body weight for the restriction group was 1.75 ± 0.21%. Gender had no influence on this response.

Body composition and urinary nitrogen excretion

It appears that a majority of the weight loss in the energy restriction group was in the form of lean body mass. By the end of the study lean mass was reduced by 0.84 ± 0.23 kg in the energy restriction group, while control subjects lost an average of 0.07 ± 0.39 kg ($p = 0.093$). Likewise, there was a trend for a greater increase in urinary nitrogen excretion in the energy restriction group, and when corrected for creatinine excretion, this difference approached significance (Table **4**; $p = 0.089$). Body fat in all subjects was stable throughout the baseline and intervention periods. Again, gender had no influence on these results.

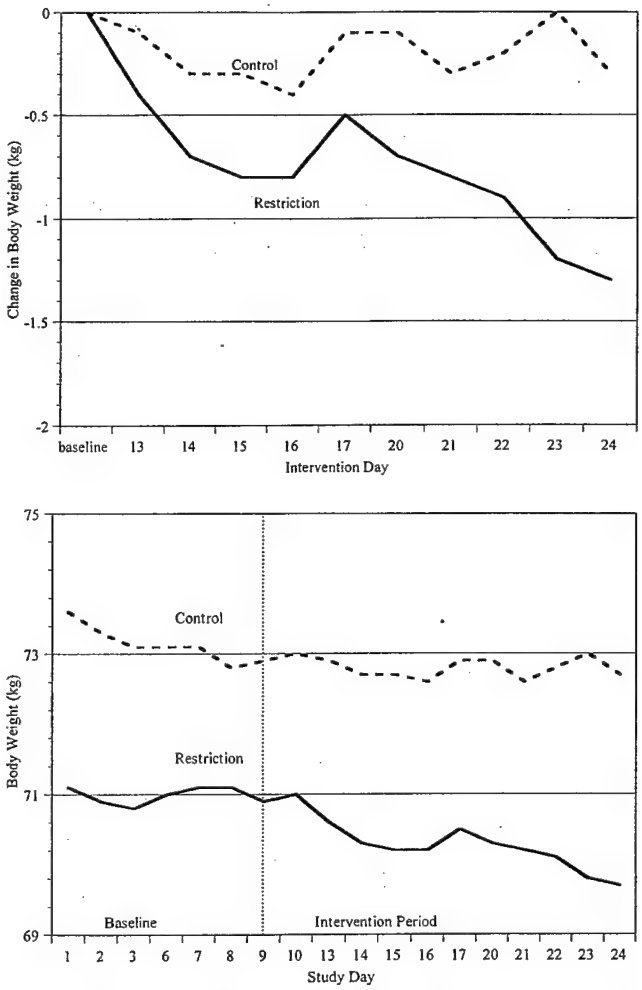

Fig. 2 Mean body weight change from baseline (a) and absolute body weight (b) for the control and energy restriction groups. There was a significant treatment × time interaction indicating greater weight loss in the energy restriction vs. control group, $p < 0.001$.

Table 4 Body composition and urinary nitrogen excretion during the baseline and intervention periods

	Restriction Group		Control Group	
	Baseline	Intervention	Baseline	Intervention
Lean Body Mass (kg)	51.9±1.8	51.1±2.7*	55.7±4.1	55.6±3.9
Fat Mass (kg)	16.6±1.7	16.1±1.6	16.2±2.5	15.9±2.5
Nitrogen Excretion (g/24 hr)	10.4±1.0	13.2±1.0	10.6±1.0	11.2±1.0
Creatinine Excretion (g/24hr)	1.56±0.12	1.54±0.11	1.48±0.06	1.59±0.13
Nitrogen/Creatinine (per 24 hr)	6.83±0.48	8.50±0.41#	7.19±0.51	7.21±0.41

Results are presented as mean ± SE. There was a trend for greater reduction in lean body mass in the energy restriction group (*$p = 0.093$). When corrected for creatinine, nitrogen excretion also tended to be higher in the energy restriction group following the intervention period (#$p = 0.089$).

Exercise performance

Energy restriction did not impair measures of 5 mile run time or muscle endurance. In fact, when compared with baseline measures, both were significantly improved after energy restriction or control. Shoulder strength was unchanged in either group, but small yet significant improvements in leg muscle strength were observed after energy restriction and control. Total work during the Wingate test declined in the control (−649 ± 288 joules) and increased in the energy restriction group (+368 ± 219 joules). This difference was statistically significant ($p < 0.05$). The same was true when total work was expressed per kg body weight. For example, relative work decreased from 230 ± 13 to 222 ± 15 joules/kg in control and increased from 207 ± 7 to 216 ± 7 joules/kg in the energy restriction ($p < 0.01$). Values for all exercise performance tests are presented in Table **5**.

Discussion

The results of this study demonstrate that in exercising men and women, two weeks of moderate energy restriction does not impair skeletal muscle strength, endurance or 5 mile run time, and may even improve anaerobic capacity. This implies that physically active men and women can become accustomed to, over the short-term, a moderate imbalance between energy intake and expenditure without sacrificing capacity to perform aerobic as well as anaerobic exercise tasks. However, energy restriction did result in modest weight loss, with a majority coming from the lean body mass compartment.

We conducted a detailed and well-controlled feeding study that incorporated commonly available foods in a variety of forms (fresh, frozen, canned and dried) to establish an imbalance between energy intake and expenditure. Adherence to the diet and exercise regimen was excellent. Thus, we feel the lack of a negative effect of energy restriction on exercise performance is a robust finding and not a consequence of poor compliance to treatment. Further, results from other studies are in general agreement with ours. Mourier et al. [12] reported that muscular strength, endurance and anaerobic capacity of competitive wrestlers was not altered following 19 days of hypocaloric feeding. Parkes et al. [13] have recently shown that quadriceps muscle function of females athletes was not reduced after 14 days of energy restriction, which resulted in a caloric intake equivalent to 75% of weight maintenance intake.

Several studies have been conducted using strength and power athletes to investigate the effect of weight loss on anaerobic performance [8,11,14]. Compared to our study, these were of shorter duration (4 to 7 days) and employed greater energy restriction (i.e., >1000 kcal/d). The results indicate that substantial weight loss induced by severe energy restriction could decrease anaerobic power but carbohydrate content of the restrictive diet seems to modulate this response.

When carbohydrate content of the energy restrictive diet was high (3.5–4.0 g/kg) anaerobic performance was maintained. When it was low (i.e., <3.0 g/kg) anaerobic performance declined. Thus, in strength and power athletes performance decrements seem to be related more to reduced carbohydrate content of diets than to decreased energy intake.

We were surprised to find that 5 mile run time did not get worse following energy restriction, and that in fact it was improved in almost every subject by the end of the intervention. Based on calculated running velocities during the performance tests, we have estimated that subjects were working at approximately 75–80% of max $\dot{V}O_2$. Since glycogen depletion is a limiting factor in activities that require 65–85% max $\dot{V}O_2$ [3], it seems likely that stored carbohydrate (muscle and liver glycogen) was a preferred substrate during the test. Subjects in the energy restriction group were receiving ~3.7 g/kg of carbohydrate while controls were receiving ~5.2 g/kg. While daily carbohydrate intake was significantly less in the restriction group, it was still above the 3.0 g/kg carbohydrate intake that appears necessary to maintain anaerobic performance in energy restricted strength and power athletes [8,11,12,14]. Thus, it may be that carbohydrate intake in the restriction group maintained sufficient carbohydrate stores to meet the energy demands of a 5 mile performance run.

We did not systematically acquire subjective ratings of perceived exertion or fatigue at any time during the experiment. One reason was that we did not want to prompt subjects to

Table 5 Exercise performance results during baseline and following intervention

	Restriction Group		Control Group	
	Baseline	Intervention	Baseline	Intervention
Muscle Endurance (repetitions)	73 ± 16	88 ± 19 Δ = 14 ± 6	69 ± 10	95 ± 29 Δ = 26 ± 21
5 Mile Run Time (seconds)	2566 ± 72	2380 ± 54 Δ = −186 ± 49	2612 ± 88	2475 ± 77 Δ = −136 ± 54
Leg Muscle Strength (kg)	135.5 ± 9.1	140.9 ± 9.5 Δ = 11.9 ± 5.1	147.3 ± 13.2	150.0 ± 12.7 Δ = 6.2 ± 7.8
Shoulder Muscle Strength	47.7 ± 6.4	48.2 ± 6.8 Δ = 0.6 ± 1.7	64.1 ± 11.4	64.1 ± 12.3 Δ = 0.0 ± 3.3
Anaerobic capacity (Joules)	14782 ± 900	15150 ± 928* Δ = 367 ± 219	17032 ± 1603	16384 ± 1697 Δ = −649 ± 288

Data are presented and mean ± SE. There were no significant effects of energy restriction on muscle endurance, 5 mile run time, or muscle strength. After the intervention period anaerobic capacity, expressed as total work (in joules) accomplished during the Wingate test, increased in the energy restriction group while a decrease was observed for control (*$p < 0.05$). Repeated measures ANOVA revealed a significant ($p < 0.05$) main effect of time for muscle endurance, 5-mile run time, and muscle strength indicating that performance was improved in all subjects when measured during the experimental period

think about which dietary condition they had been randomized to. Nonetheless, it was interesting to note that a majority of unprovoked, self-reports of muscular fatigue, soreness and lethargy were made toward the end of the baseline period. While a majority of the subjects were considered to be recreational runners, none could be classified as "well-trained". Thus, daily treadmill running which began at the start of the baseline period may have represented a sudden increase in frequency (and volume) of training for many of the subjects. This could have resulted in an inability to perform well during tests of muscular endurance and 5 mile run time and a "true" measure of baseline performance was not obtained. Subsequent testing was performed after there had been adequate time to adapt to the frequency and volume of training. Perhaps, what appeared to be an increase in performance was actually a normalization to "true" baseline abilities. Even if this were so, energy restriction did not appear to prevent adaptation to the increased training load. Further, the data could be interpreted to suggest that the sudden increase in training load was more detrimental to performance than moderate energy restriction during adaptation to such training. Additional experiments are planned to test these hypothesis.

Two weeks of moderate energy restriction resulted in nearly a 2 kg weight loss and a majority of this loss came from lean body mass. Despite a decrease in lean body mass, exercise performance was either maintained or increased in the energy restriction group. There are few well-controlled studies on the effects exercise and moderate energy restriction have on body composition of athletes, amateur or recreational. Mourier et al. [12] reported that hypocaloric dieting in trained wrestlers reduced thigh skeletal muscle cross-sectional area, as determined by magnetic resonance imaging. Similar to our findings, muscle performance was maintained in spite of reduced muscle mass. They also reported that varying the protein content or enriching the hypocaloric diet with branched chain amino acids did little to prevent loss of muscle area. In our study, we maintained protein intake at ~1.4 g/kg during energy restriction but this apparently had little effect on maintenance of lean body mass or urinary nitrogen balance. Sophisticated protein turnover studies are needed to specifically determine if energy restriction disrupts dynamic muscle protein balance in aerobically training men and women. Regardless, loss of lean tissue during exercise with energy restriction should be prevented because a slow but persistent loss would ultimately disrupt exercise performance.

The anaerobic capacity results are perplexing. As already mentioned, daily treadmill running resulted in a significant increase in the frequency and probably volume of training in our recreational runners. Thus, it is likely that muscular adaptations, which helped to meet the demands of the increased aerobic training load, were made but these changes did little to enhance the muscle's capacity to tolerate anaerobic type activities and may have even decreased anaerobic capacity [7]. On the other hand, energy restriction during this adaptive phase seemed to prevent such a decline in anaerobic performance. Clearly, this observation and interpretation deserves further testing, particularly with special attention to biochemical and morphological changes in skeletal muscle of men and women undergoing aerobic training with energy restriction.

It is not unreasonable to think that recreational or amateur athletes engaged in intensive training could be operating under conditions of energy imbalance, mostly due to inappropriately low energy intake. Reasons for a weak coupling between energy expenditure (exercise) and intake are not well-defined, but could be related to lack of time for meal preparation and consumption or a reduced drive to eat due to the stressful nature of training. In this light, the findings of the present investigation have practical implications for athletes and suggest that physical performance is not negatively affected by short-term, moderate (750 kcal/d) energy restriction, despite loss of body weight. Nonetheless, the finding also suggest that a short-term imbalance between energy intake and expenditure in aerobically training athletes should be avoided because there is the potential for significant decrements in lean body mass.

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TASK V

NUTRIENT DATABASE INTEGRATION LABORATORY

NONE

TASK VI

ENHANCING MILITARY DIETS

TASK VI

Appendix A

Manuscript from Study 1 that is being revised for publication in *Military Medicine*

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Changes in Food Intake and Body Weight Associated with Basic Combat Training

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Abstract

This research project evaluated changes in food selections, food intake, and body weight during Basic Combat Training (BCT). During the first week of BCT, 139 soldiers from two Companies volunteered for participation in the study. In the eighth week of BCT, 92 soldiers were available for retesting. A digital photography method for measuring food selections and food intake was developed for this study. Fruit intake of soldiers was very low at the beginning and end of BCT. Food intake for grains and milk products were low during the first week of BCT, but improved by the end of BCT. Average body weight decreased over the eight weeks of BCT, but heavier soldiers tended to lose weight and thinner soldiers tended to gain weight. These findings suggest that the overall effect of BCT was a trend toward improvement of healthy eating and healthy body weight.

Changes in Food Intake and Body Weight Associated with Basic Combat Training

One goal of the military is the promotion of healthy eating by soldiers.[1,2] The Committee on Military Nutrition Research of the Institute of Medicine recommended that soldiers consume adequate amounts of food in relation to the physical activity required by military service, as well as increase consumption of foods that provide good sources of antioxidant nutrients (e.g., vitamins A, E, and C), calcium, and folate.[3,4] The best food sources for these nutrients are fruits, vegetables, grains, and milk products. A number of population-based studies support the conclusion that consumption of fruits, vegetables, and grains may be protective for coronary heart disease[5,6] and cancer.[7]

In this study, we studied the food selections and food intake of soldiers in Basic Combat Training (BCT). Soldiers in BCT were selected for study for three primary reasons: 1) the energy requirements of BCT are high because of the high level of physical activity with subsequent intense nutrient demands, 2) BCT is the introductory career stage for Army soldiers, and 3) soldiers consume food only from the dining facilities (DFACS) during BCT. This final point is significant because there are few military environments where one can directly observe 100% of food selections and intake.

Based upon these considerations, we aimed to study changes in: a) total caloric intake, b) body weight, and c) consumption of fruits, vegetables, grains, milk products, meats, dietary fats, and added sugar, over the eight weeks of BCT. Finally, since the eating environment of BCT requires soldiers to move in and out of DFACs in a relatively efficient manner, i.e., less than 30 minutes, we studied the relationship between food intake and total time in the DFACs , time to select foods, and time to eat foods.

One objective of the research study was to minimally disrupt the normal operations of BCT. Another objective was to keep the identities of the research participants anonymous, since BCT soldiers represent a population at risk for coercion and therefore should be protected from repercussions for nonparticipation in research projects. To accomplish these two objectives, the research team developed several innovative approaches for gathering the research data.

Method

Participants

The soldiers of two companies from two different brigades stationed at Ft. Jackson, SC, were studied. During the first week of BCT, the soldiers were briefed about the study requirements and volunteered by providing written consent to participate in research. The only eligibility requirement was age greater than 17 years. A total of 139 soldiers volunteered for the study. This sample represented approximately 45% of the soldiers assigned to the two companies. When data were collected at the end of BCT, 92 soldiers (66% of the original sample) were available for study. Table 1 summarizes the demographic characteristics of the samples for study in Weeks 1 and 8. Despite an attrition rate of 34% (primarily drop-outs from BCT), the characteristics of the sample remained similar.

Identification of soldiers using barcode technology

One objective of this study was to maintain the anonymity of the identity of each soldier, but the study design required that we correlate data collected during two days in the first week of training with that collected during the eighth week of BCT. We developed a novel method using the barcode (a unique identifying code) printed on the

back of the soldier's military identification (ID) card as a means of anonymously tracking an individual soldier over time. Barcodes were recorded prior to the collection of all data (at any point in time) using hand-held computers that were equipped with barcode readers.

Assessment methods

Collection of timing data using hand-held computers. One objective was to measure the total amount of time that soldiers spent in the DFACs, time for selecting foods, and time to eat. To accomplish this objective, handheld computers equipped with barcode scanners (Symbol SPT 1500) collected timing data at four points in each of the two DFACs. At location 1 where the soldier first entered the DFAC, a researcher scanned the military ID and issued a bar-coded clip-on badge that was also scanned. By concurrently scanning the soldier's ID card and the clip-on badge, the two bar codes were linked for later retrieval, thus enabling the tracking of movement of individual soldiers. The soldier carried this clip-on badge to location 2 at the beginning of the serving line. Next, a second researcher scanned the barcode of the clip-on badge and attached it to the soldier's food tray. Bar codes were then scanned at two other locations: location 3, a point where the soldier had completed food selection, and location 4, after the meal had been consumed. The clocks of the computers were synchronized and were used to record the time at which the soldier arrived at each of the four locations. Using subtraction, the following timing variables were measured: 1) length of time the soldier spent in line before reaching the serving line (Location 2 minus Location 1), 2) time that the soldier spent in the serving line (Location 3 minus Location 2), 3) time spent eating (Location 4

minus Location 3), and 4) the total time spent in the DFAC (Location 4 minus Location 1).

Digital photography of food intake. Another technology, developed to measure food selections and food intake, was digital photography of food trays before and after eating. A digital video camera was used to capture photographs of each soldier's food selections and, after eating, food that was uneaten. We chose a digital video camera because it produces very rapid photographic measurement (i.e., thirty frames per second). This technique was developed to minimize the impact of our study on the soldier's eating behavior while enabling measurement of individual soldier's food selections and intake.

To control for differences in menus across the two measurement periods at the beginning and end of BCT (two days in Week 1 and two days during week 8 of BCT), the DFACs served identical foods during the two periods of measurement. Prior to recording the food selections of each meal, dietitians studied recipes and food preparation for each food on the menu. Also, standard servings (from the food service delivery line) for each food were quantified using measures of weight and/or volume. Digital photographs of these standards were then collected for later comparison to the foods on each soldier's tray.

A digital video camera was located at a centralized location in the DFACs between the point at which soldiers exited the serving line (Location 3) and before they entered the tray disposal station (Location 4). Over the duration of the study, the camera lens was positioned .62 m (24.5 inches) above the tray with a camera angle of approximately 45 degrees. At location 3, the soldier's tray was placed under the video

camera and photographed. The same procedure was followed when the soldier had finished eating (at Location 4) to capture the amount of food that was uneaten.

The digital video camera was connected to a computer and the picture of the soldier's tray that had greatest clarity was captured using a video capture board. Three research associates who had been trained to use the visual estimation method for estimating food intake[8], classified food selections, amount of food selected, and amount of food consumed. Foods were classified using the USDA food grouping system of the Food Guide Pyramid.[9] Foods were classified as: fruits, vegetables, grains, milk products, meats, added sugar, and dietary fat. Dietary fat and added sugar were classified in grams and meat in ounces. All other food categories were classified in terms of Food Guide Pyramid serving sizes. Total daily caloric intake was estimated using the following caloric values for each Food Guide Pyramid Serving: 1) one ounce meat = 35 kcal, 2) milk serving = 90 kcal, 3) fruit serving = 60 kcal, 4) vegetable serving = 25 kcal, 5) grain serving = 80 kcal, 6) one gram of fat = 9 kcal, and 7) one gram of added sugar = 4 kcal. Food standards were established (using Food Guide Pyramid guidelines) by measured volume or weight and were photographed. The food analysis program allowed the dietitians to directly compare the foods on a soldier's tray with these food standards displayed on the same video screen.

Photo captures were oversampled by 20% as a means of checking reliability/accuracy of food classifications and estimates of amounts of foods selected and consumed, for the three research associates. Comparison of the foods classified by the three dietitians indicated that they agreed greater than 80% of the time for all specific foods that were classified, e.g., potato, chicken, bacon, sausage, egg, etc. The rate of

agreement for classifying most foods was very high (> 95%), but condiments, butter, and foods with similar appearance, e.g., turkey and pork bacon, were more difficult to distinguish and had lower rates (80% to 95%) of agreement. On average, soldiers generally consumed about 87% of the foods that they selected. Men consumed an average of 91% of the foods that were selected and women consumed an average of 82% of foods that were selected. The three research associates had a very high degree of agreement pertaining to portion sizes before eating and plate waste after eating, and the variance attributed to variability in the estimates of the individual research associate was insignificant compared to the overall variance. Furthermore, the correlation between the amount of food selected within a category and the amount of food consumed within the same category (for the same meal), was very high (all r values > .98) for all categories. Given the very high correlation between food selections and food intake, the findings pertaining to food intake were selected for presentation.

Body Mass Index. The height and weight of each soldier (in uniform) were measured using balance beam scales and a stadiometer. These measurements were converted to body mass index (BMI = kg/m^2), which is a measure of body mass that controls for height. Normal values of BMI are values between 20 and 25. Values above 25 are considered to be overweight and BMI values above 30 define obesity in adults.[10]

Procedure

The research protocol was approved by the Institutional Review Boards of Pennington Biomedical Research Center and the Human Subjects Review Board of the U.S. Army. Data were collected during two days of Week 1 of BCT and during two days of Week 8 of BCT. The BCT schedule of the two companies was spaced one week apart,

which allowed for the collection of data during parallel training periods for the two companies. Demographic and height/weight data were collected in a one-hour assessment session with each of the two companies. Timing variables and digital photography of the soldiers' food selections and food intake were collected at breakfast, lunch, and dinner of each day.

Results

Analysis of the Digital Photographs of Foods selected and consumed

Percentage of soldiers who met Food Pyramid Guidelines (FPG). The average food intake for each soldier was compared to the recommended number of daily servings for "active men" and "very active women" as defined in the USDA's Food Guide Pyramid.[9] Table 2 summarizes the FPGs for each food category. Changes in the percentage of soldiers meeting these FPG criteria at Weeks 1 and 8 were tested using the McNemar test. Very few soldiers consumed four or more servings of fruit at the beginning or at the end of BCT and the percentage of soldiers consuming adequate amounts of fruit did not improve over the eight weeks of BCT. At the beginning of BCT, most soldiers consumed an adequate number of vegetable servings and vegetable intake did not change significantly from Week 1 to Week 8 ($p > .05$). In Week 1, few soldiers consumed adequate amounts of grain. By the end of BCT, however, the number of soldiers who met the FPG criteria for grains improved significantly ($p < .001$). A similar improvement in the number of soldiers meeting the FPG criteria for consumption of milk products was also found ($p < .007$). The soldiers generally exceeded the FPG for meat, dietary fat, and added sugar. The percentage of soldiers who met the standards for lower intake of dietary fat decreased from week 1 to week 8 ($p < .01$). Two different calorie

levels (exceptionally high and high activity) were selected for study based upon military dietary reference guidelines. A majority of soldiers were meeting all of these caloric goals during Week 8 and the percentage of soldiers meeting each of these caloric goals increased from week 1 to week 8 ($p < .0001$).

Average number of servings for food selections and food intake

Changes in food intake were analyzed using Analysis of Variance with the independent variables: Company, Gender, Ethnicity, and Time. Changes in food selections and food intake from Week 1 to Week 8 of BCT are summarized in Table 3.

Fruit. The average number of servings of fruit that were selected and consumed was well below the FPG of four servings per day. Fruit selections increased significantly from Week 1 to Week 8, but the actual increase in servings was quite small, i.e., only about 1/3 serving. Statistical analyses indicated a significant Gender X Time interaction for fruit intake, $F(1,122) = 6.77$, $p < .01$. Female soldiers, but not male soldiers, increased fruit intake from Week 1 to Week 8 of BCT.

Vegetables. On average, the intake of vegetables decreased over the eight weeks of BCT. Men ($M = 5.78 \pm .22$ servings) consumed, more vegetable servings in comparison to women ($M = 4.98 \pm .22$ servings) , $F(1,122) = 20.94$, $p < .003$.The average intake of vegetables exceeded the FPG of 5 servings per day at Week 1, but was slightly below this FPG at Week 8.

Grains. Average grain intake increased significantly from Week 1 to Week 8. In Week 1, soldiers were not consuming the FPG of 11 grain servings/day, but by the end of BCT, this standard was met, on average. Male soldiers consumed more grain servings in comparison to women, $F(1,122) = 31.85$, $p < .0001$. The mean grain intake of men

was 12.03 ± .45 servings/ per day. In comparison, women consumed 9.87 ± .47 servings on average, which was below the FPG of 11 servings per day.

Milk products. Average milk intake increased from well below the FPG of 3 servings/day at Week 1 to near that standard at Week 8. Also, African-American soldiers consumed fewer milk products (mean = 1.98 ± .32 servings per day), on average, than Caucasian soldiers (mean = 3.17 ± .21 servings per day).

Meat. Meat intake was well above FPG standard of 7 ounces/day and did not change significantly over time. For meat consumption, a significant effect for gender was found, F (1,122) 13.88, $p < .0003$; males ate significantly more meat in comparison to women, averages of 11.05 ± .38 ounces per day versus 9.01 ± .39 ounces per day.

Dietary fat. Average fat intake exceeded the FPG standard of 93 grams per day and increased significantly from Week 1 to Week 8. Women consumed significantly less fat than men, F (1, 122) = 5.73, $p < .02$; with average intakes of 126.99 ± 3.76 grams/day for men and 113.95 ± 3.94 grams/day for women.

Added sugar. Sugar intake was very high in Week 1and increased further from Week 1 to Week 8.

Total Calories: Estimated daily total caloric content of foods selected and consumed were analyzed using Analysis of Variance with the following independent variables: Company, Gender, and overweight status (defined as a BMI > 25). During Week 1, overweight soldiers selected foods with significantly lower calories, F (1.124) = 4.51, $p < .04$. Overweight soldiers selected foods averaging 3453 ± 105 kcal/day; normal weight soldiers selected foods averaging 3724 ± 72 kcal/day. These two groups of soldiers did not differ in caloric intake, however. Reduction in body weight (kg lost) was

correlated with total caloric intake during Week 1 (r = -.32) and Week 8 (r = -.22). These correlations indicate that weight loss was associated with lower caloric intake.

The caloric intake of women was significantly lower than for men, F (1, 124) = 8.43, $p < .004$, with women consuming an average of 3016 ± 100 kcal/day and men consuming an average of 3386 ± 79 kcal/day. On average, total caloric intake increased from Week 1 to Week 8, F (1,122) = 65.72, $p < .0001$; the mean increase was 671 kcal/day.

Environmental Factors associated with Food Selections

On average, soldiers were in and out of the dining facilities in less than 30 minutes and the total amount of time for each meal was relatively stable across the four days of measurement. Average total time across the four days varied as a function of meal type during Week 1, F (2, 226) = 66.64, $p < .0001$. The length of all three types of meals differed significantly. Time required for Breakfast was shorter (mean = 20.1 ± .22 minutes) in comparison to the time required for lunch (mean = 25.5 ± .33 minutes), and dinner (mean = 23.1 ± .22 minutes). The correlations of timing variables with food selections indicated that, during Week 1, time available to eat foods was positively correlated with total caloric intake. This association was found for breakfast (r = .45), lunch (r = .22), and dinner (r = .29). These correlations were not significant at Week 8, however.

Body Weight Changes during BCT

On average, BCT soldiers lost 2.29 ± .67 kg during the eight weeks of BCT. This weight loss varied as a function of gender and ethnicity. Table 4 summarizes the mean changes in body weight of men and women in three ethnic groups. In this sample,

Caucasian and "other minority" males and black females lost the most weight (3 to 4 kg) relative to the other groups, who lost an average of less than 1 kg. Figure 1 illustrates the relationship between BMI at Week 1 and BMI at Week 8. In this figure, each soldier is depicted by one data point and the solid line indicates no increase or decrease of BMI from Week 1 to Week 8. The dashed line illustrates the regression line for the correlation ($r = .91$) between BMI at Week 1 and Week 8. As can be seen, the trend was for soldiers with a BMI > 23 at Week 1 to have a reduced BMI at Week 8 and for soldiers with a BMI < 23 at Week 1 to have an increased BMI at Week 8. In general, one effect of BCT was to "normalize" BMI. In fact, all soldiers in this sample with an initial BMI > 29, lost weight during BCT.

Discussion

This study found that the overall effect of BCT was a trend toward improvement of healthy eating. Furthermore, overweight soldiers lost weight and thin soldiers gained weight. In this study, changes in body composition were not measured, but we believe thin soldiers and overweight soldiers probably lost body fat and gained lean body mass. Other studies of body composition changes during BCT (in Britain and Singapore) have reported this pattern of change in body composition to be associated with an overall reduction in body weight.[11,12,13]

During Week 1, on average, the food intake of soldiers did not meet FPGs for fruit, grain, or milk. Thus, upon entering BCT, most soldiers were not consuming adequate amounts of food that are good sources of antioxidant nutrients, folate, and calcium, which reinforces the concerns raised by the Committee on Military Nutrition Research of the Institute of Medicine.[4] The food intake of soldiers at the beginning of

BCT is quite consistent with the pattern of nutrition of most American adults[14,15,16] and adolescents.[17] However, in Week 8, with the exception of fruits, the consumption of most food groups had improved. Most soldiers were meeting the Army guidelines for caloric intake. This finding is encouraging given recent concerns that soldiers may not be eating adequate calories relative to the physical demands of BCT.[2]

Several technical innovations related to data acquisition were developed. The digital photography method for measuring food selections and food intake was found to have very good reliability/agreement across observers. This new method allowed the research team to gather data relatively quickly and unobtrusively, with minimal disruption to the normal routine of the two military dining halls. There are many problems associated with the measurement of food intake using self-report methods.[18] One alternative to self-report methods is the visual estimation procedure[19,20,8] in which human observers estimate portion sizes and/or volume of foods before and after eating. Since the visual estimation procedure requires human observers to directly examine foods on a participant's plate in the dining facility, it can be time consuming, intrusive, and disruptive. The digital photography method, an alternative to the visual estimation method, lacks these disadvantages.

A second innovation was the use of palm-held computers to track the data of individual soldiers over time and environments while maintaining anonymity of the soldier's identity. Palm-held computers were also used to measure movement through the serving lines of the DFACs. We found that the palm-held computers with barcode readers served these two functions with very little disruption of the soldiers' routine activities.

On average, women did not meet the FPG of 5 vegetables/day in Week 1 or in Week 8. Also, during Week 8, the average grain intake of women was below the FPG, i.e., 11 servings/day. Neither men nor women consumed adequate amounts of fruit. Recent studies of civilians have reported similar findings, that women are at higher risk for underconsumption of fruits and vegetables.[14,16]

African-Americans consumed less milk than Caucasians, which was the only ethnic difference that was found. This finding is consistent with the findings of a recent study of Air Force recruits.[21] African-American women and other minority males lost more weight than most other groups. This finding is encouraging given the finding that minority populations are at significant risk for the development of chronic obesity.[22]

Overweight soldiers restricted caloric intake during the first week of BCT. Overweight status is strongly discouraged within the military community[23] and overweight recruits are told that they will need to lose weight in order to stay in the military.

In conclusion, this study found that the BCT experience generally produced positive behavioral and health outcomes related to nutrition and body weight. The most conspicuous problems are very low fruit intake by men and women and low vegetable, milk, and grain intake in women. Also, African-Americans did not consume adequate amounts of milk products. Therefore, we can presume that the intakes of the antioxidant nutrients, folate, and calcium are deficient in male and female soldiers in BCT.

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for the Study of Obesity, Long Beach, CA, November 1, 2000.

Table 1. Description of the study sample

		Week 1 (n=139)	Week 8 (n=92)
Height (m)	M=	1.73	1.74
	SD=	0.09	0.09
Weight (kg)	M=	72.93	74.70
	SD=	12.34	12.31
BMI (kg/m^2)	M=	25.13	25.33
	SD=	3.29	3.27
Age (yr)	M=	20.91	21.04
	SD=	3.19	3.39
Males		55.8%	58.7%
Females		44.2%	41.3%
Caucasian		55.0%	57.6%
African-American		26.4%	23.9%
Other Minority		18.6%	18.5%
% overweight (BMI >25)		36.4%	24.2%

Table Note: Abbreviations are: m = meters, kg = kilograms, yr = year, BMI = body mass index

Table 2. Percentage of soldiers who met the FPG's for food intake at Week 1 and Week 8 of BCT

Food Category	Recommended Servings	Week 1	Week 8
Fruit	4 or more servings/day	3.0%	5.1%
Vegetables	5 or more servings/day	57.6%	52.5%
Grain	11 or more serving/day	23.5%	61.6%
Milk	3 or more servings/day	28.0%	49.5%
Meat	7 or more ounces/day	84.1%	87.9%
Dietary Fat	93 g. or less/day	14.4%	2.0%
Added Sugar	72 g. or less/day	1.5%	1.0%
Total Calories:			
E H Activ.	Men= 4600 kcal/day or more		
	Women=3150 kcal/day or more	41.7%	63.7%
H Activ.	Men=3950 kcal/day or more		
	Women=2700 kcal/day or more	61.4%	86.9%

Table Note: Abbreviations are: E H Activity = Extremely High Activity, H Activity = High activity, kcal = kilocalories

Table 3. Average food intake (number of servings) at Week 1 and Week 8 of BCT

Food Category	Recommended Servings	Week 1	Week 8	F value
Fruit	4+ servings/day	1.52 ± .10	1.78 ± .14	3.62
Vegetables	5+ servings/day	5.78 ± .22	4.98 ± .22	9.29**
Grain	11+ servings/day	9.69 ± .37	12.21 ± .42	31.85***
Milk	3+ servings/day	1.80 ± .16	2.93 ± .21	33.90***
Meat	7+ ounces/day	9.98 ± .32	10.08 ± .34	.07
Dietary Fat	93 g. or less/day	107.79 ± 3.02	133.14 ± 3.62	43.49***
Added Sugar	72 g. or less/day	171.36 ± 4.97	185.70 ± 5.84	5.38*
Total Calories (kcal)	3178 ± 66.15	3849 ± 72.23	65.72***	

Table Note: * =p < .05, ** = p < .005, *** = p < .001

degrees of freedom for all ANOVA's was 1,122.

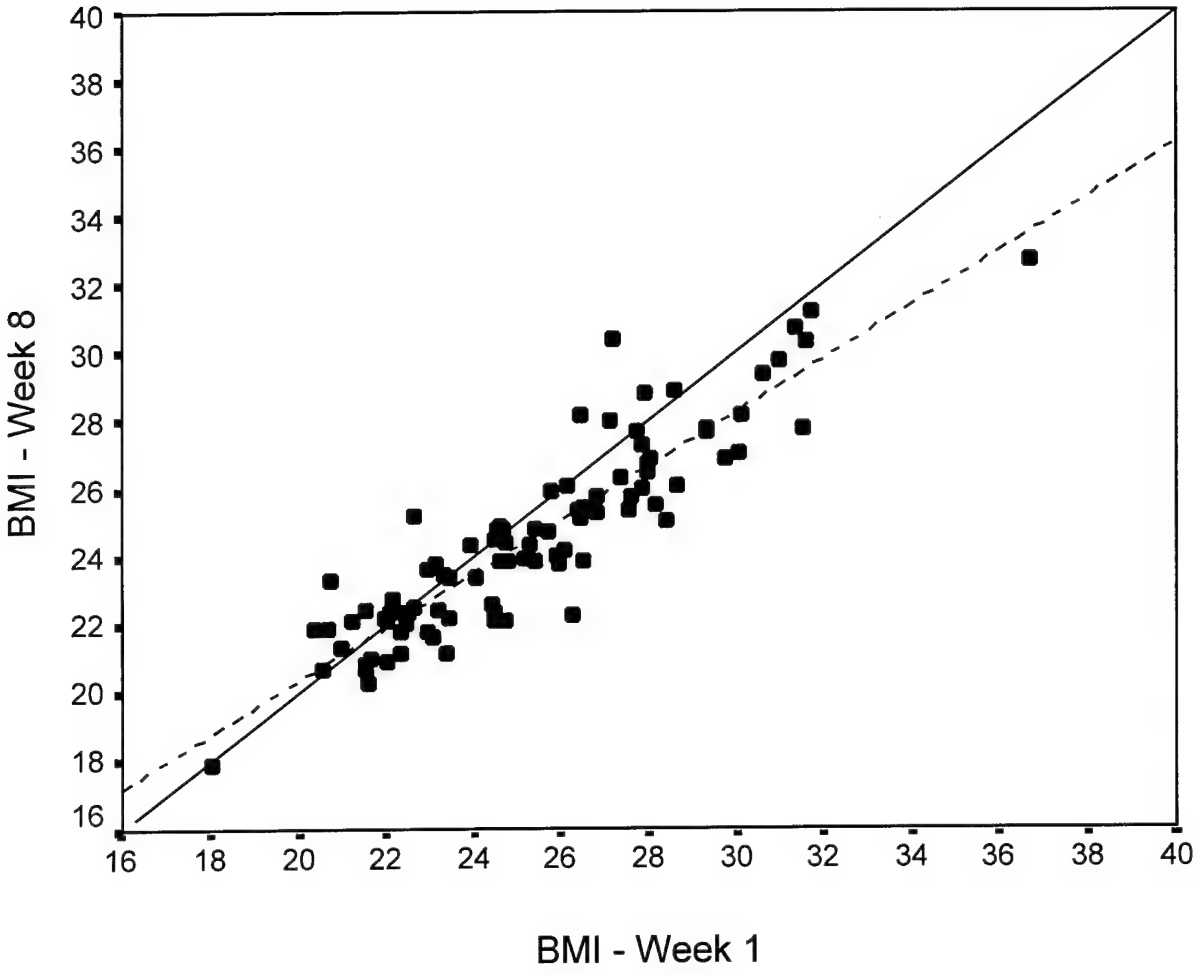
Table 4. Weight loss (kg) during BCT as a function of Gender and Ethnicity

		Caucasian	African-American	Other Minority
Male	M=	3.01[a]	.66[b]	4.09[a]
	SD=	4.38	3.88	4.35
Female	M=	.35[b]	3.50[a]	.88[b]
	SD=	3.99	2.37	1.75

Table note: Superscripts (a,b) that are different indicate means are significantly different ($p < .05$).

Figure Caption

Figure 1. Scatterplot of BMI at week 1 of BCT with BMI at week 8 of BMI. Each data point represents an individual soldier. Individuals above the solid line gained weight during BCT. Soldiers below the solid line lost weight during BCT. The dashed line shows the regression plot for the data set


BMI - Week 8
BMI - Week 1
40
38
36
34
32
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26
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TASK VI

Appendix B

Manuscript that describes the development of the Military Stress Scale

Pages: 17
Words: 2,957
Tables and Figures: 4
Photos: 0
References: 13
Contact: P.D. Martin
Guarantor: P.D. Martin

DRAFT

The BCT Military Stress Scale:

Reliability, Validity and Scale Development

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Key words: Stress, basic combat training

Military Stress Scale

Abstract

This research project developed a measure of stress during Basic Combat Training (BCT). During the first week of BCT, 139 soldiers from two Companies volunteered for participation in the study. In the eighth week of BCT, 92 soldiers were available for retesting. Stress is frequently associated with major changes in lifestyle. While there is a substantial body of literature investigating stress in numerous life situations the changes associated with the transition from civilian to military living during BCT has been overlooked. The present study reports the development and preliminary reliability and validity studies of the Military Stress Scale (MSS). This 28 item likert scale assesses the degree of stress associated with various aspects of military training. Methods used to construct the subscales, measuring different dimensions of military training, include item analyses and principal components factor analysis. Results indicate that the MSS is psychometrically sound and may be a useful tool for assessment of degree of stress experienced by military personnel during basic combat training.

Introduction

Initial entry training (IET) is the basic combat training experience designed to transition soldiers from civilian to military life. While most would agree that the experience of IET is stressful, to date, few studies have provided systematic investigation (Gold & Friedman, 2000; Clemons, 1996;). Previous studies of stress in military environments have determined that lists of stressors unique to the experience being evaluated are necessary (Slusarcick, Ursano, Fullerton & Dinneen, 1999). Currently available stress measures failed to capture the types of unique stressors encountered on a daily basis during basic training; therefore there is a need for an objective measure of this type of stress.

The Military Stress Scale (MSS) was designed to provide a reliable and valid measure of stress experienced by initial entry students during basic combat training. It was designed to assess the unique experiences of the individual entering military living for the first time which greatly differs from the traditional stressors experienced by other populations in a free living civilian environment. It was based on the daily experiences of the trainees and designed to provide programmatically useful information regarding leading areas of stress. The degree of item specificity should provide the structure needed to reflect the unique experiences of these individuals and therefore, a better conceptualization of stress.

Because IET students are closely monitored and may be uncomfortable admitting their level of distress, a multiple response format was included so that soldiers could communicate stress through degrees of agreement. Therefore, the MSS should improve upon currently available stress measures by providing a micro measure of the basic combat training student's

unique experience. Since adaptation to stress is a necessary for optimal readiness and effectiveness for any unit (Pruitt & Bernheim, 1991) this instrument may be of programmatic usefulness in identifying individuals at risk for poor adaptation.

Study One: Field Trial

Method

Construction of initial item pool

The scale was developed following the steps outlined by Crocker and Algina for likert scales (1986). The initial step involved a review of the stress literature and current assessment instruments as well as the experiences and knowledge of an interdisciplinary team of military personal and behavioral psychologists. Items were selected that were relatively minor events that had a high potential for occurring in any given week. Items were also chosen to represent an observable event with a discrete beginning and end (Zatura et al., 1986)

A 5-point likert scale was used to assess the degree of agreement on each individual statement. The scale was modeled after the Weekly Stress Inventory (WSI). The scale was reviewed for content validity by a team of military personnel and two clinical psychology faculty members. Analysis of word length and sentence complexity using Grammatik 5 software indicated that the MSS requires an 8th grade reading level. Prior to piloting, the items were again reviewed and assessed for bias, accuracy, appropriateness, and grammar.

Field trial

Once the preliminary version of the MSS was constructed, it was administered to students during their first 2 weeks of BCT who were recruited from one company of basic

combat trainees assigned to kitchen detail. A small number of soliders (N= 21) completed the preliminary scale to assess ease of readability, face validity, and adequacy of the scale format and content. The preliminary version contained 25 statements about experiences on a 5-point Likert scale ranging from 0 'not at all stressful' to 4 'very stressful'. Open-ended items were also included to obtain the patient's perspective of services that may have been missed in the item pool. Additionally a 5 item questionnaire was developed to assess the degree of overall stress from 1st week of BCT, identify stressful aspects of BCT, physiological symptoms experienced and degree of ability to cope.

During the assessment the soliders were checked periodically to answer questions or assess for difficulty completing the measures (i.e., confusion). After completion, the researchers screened for missing information and conducted brief focus groups aimed at improving content and answering subject questions. No difficulties were noted in the soldier's ability to read questions, follow scale format, or understand the purpose of the questionnaire. Based on information obtained during this session 3 items were added to improve the scope of the stressors measured.

Results from the stress questionnaire completed by 19 of the students indicated that 37% rated the first week of BCT as very stressful, 26% rated it as moderately stressful, 21% as slightly stressful and 16 % did not report the first week as stressful. The types of stressors most frequently endorsed included lack of time to complete daily tasks (e.g. eating, assignments) and group punishments for other's mistakes.

Method

Sample

The soldiers of two companies from two different brigades stationed at Ft. Jackson, SC, were studied. During the first week of BCT, the soldiers were briefed about the study requirements and volunteered by providing written consent to participate in research. The only eligibility requirement was age greater than 17 years. A total of 139 soldiers (from a pool of approximately 300 soliders in the two companies) volunteered for the study. This sample represented approximately 45% of the soldiers assigned to the two companies. When data were collected at the end of BCT, 92 soldiers (66% of the original sample) were available for study. Table 1 summarizes the demographic characteristics of the samples for study in Weeks 1 and 8. Despite an attrition rate of 34% (primarily drop-outs from BCT), the characteristics of the sample remained similar.

Measures

Measures used in Study Two consisted of the final version of the MSS, a demographic questionnaire, and several measures of stress and psychological distress. The Perceived Stress Scale (PSS) (Cohen et al., 1983), a 14 item questionnaire, was used to into global cognitive perceptions of stress (e.g. feeling overwhelmed). The Weekly Stress Inventory (WSI) (Brantley, et al, 1997) an 87 item self report measure assessing the number and stressfulness of minor stressors, was used to assess the number of stress events experienced and impact of commonly experienced stressful situations in normal daily life (e.g., had to wait in line, had argument with coworker). To assess for general psychological distress the short forms of the Beck Depression

Inventory (BDI) (Citation) and Beck Anxiety Inventory (BAI) (Reference) were administered. The BDI measures both cognitive and physical symptoms commonly associated with clinical levels of depression (e.g. thoughts of suicide, problems sleeping) while the BAI measures primary physiological arousal commonly associated with states of stress and anxiety.

Procedure

The research protocol was approved by the Institutional Review Boards of Pennington Biomedical Research Center and the U.S. Army. Data were collected during two days of Week 1 of BCT and during two days of Week 8 of BCT. The BCT schedule of the two companies was spaced one week apart, which allowed for the collection of data during parallel training periods for the two companies. Demographic and stress measures were collected in a one-hour assessment session with each of the two companies.

Refinement of item pool

Based on the results of the preliminary field test a number of modifications were made to develop the final version of the MSS. Overall 3 items were added to the scale: Based on the open-ended comments of soliders. No items were deleted due to high rates of reported inapplicability.

Analysis of the final version of the MHPSS with Grammatik 5 software suggested a Flesch-Kincaid 8.9 grade reading level. Items were assessed for deletion based on poor item total correlations (<.20), and limited variability (SD <1.0) (Nunnally, 1978). All items met both of these criteria and were retained.

Results

Principal components analysis

An exploratory principal components analysis with varimax rotation was performed on the 28 item final version of the MSS. Three guidelines were used to select factors: scree plot, eigenvalues greater than one, and presence of two or more items with a loading greater than .4. There were 5 factors with eigenvalues greater than one; however the scree plot suggested a 6-factor solution. The factors were labeled in accordance with their commonalities. All of the items loaded on at least one of the 6 factors. Items loading on more than one factor were assigned based on logical fit and item scale correlations greater than .30 (Nunnally, 1978). On the 6 factors, factor loadings were above .60 on 40-100% of the items. This high factor saturation suggested that the sample size was sufficient for interpretation and stability (Guadagnoli & Velicer, 1988). The 6 factors combined accounted for 73.7 percent of the variance. See table 3 for factor loadings.

Reliability

To measure internal consistency, Cronbach's alphas were computed on the MSS. The analysis yielded a high estimate of internal consistency for the MSS-Total (.96) and the subscales, ranged from .84 to .91. These scales met the minimum criteria of .50 for making group comparisons (Helmstadter, 1964) and the standard for early research on a construct (Nunnally, 1978). The alphas obtained are presented in Table 3. To assess stability, test-retest reliability was obtained 8 weeks after initial administration on 93 soliders. (see Table 3). The MSS-Total

demonstrated adequate stability over time (r = . 65), while the subscales yielded acceptable stability coefficients ranging from r = .51 to .63.

Scale Validation

Construct validity was assessed by correlating the final version of the MSS with the WSI and PSS. The correlations between the MSS subscales, MSS-Total, and WSI-Total and PSS-Total are presented in Table 4. All correlations were significant at $p < .0001$. Overall the MSS was highly correlated with the WSI.

Screening for Confounds

An analysis of variance was performed examining the relation between the demographic and stress variables to screen for potential confounds. Sex and age effects were nonsignificant F = .409, F = .071, respectively. However, an ethnic difference between Caucasians and Hispanic/Mexican Americans was noted F = .0459, with Caucasians reporting higher levels of stress than Hispanics mean = 53 and mean = 31, respectively. In summary, none of the potential confounds appeared to have substantial impact on the stress scores.

Discussion

The present study described the development and preliminary research on the Military Stress Scale (MSS). Overall, this scale demonstrated high internal consistency as noted by high alpha coefficients. Test-retest reliability was adequate for most scales and suggested moderate stability of stress levels over a two month period. Content validity was supported by item development from a literature review and experiences of a military personnel team as well as basic trainee input. Construct validity was supported by moderate to high correlations with the

WSI a measure of specific stress and the PSS, a measure of general stress. The highest correlations were noted between the MSS general subscale and WSI. Demographic correlations demonstrated trends noted in previous research such as the differential reporting patterns between Caucasian and minority soliders. The lower correlation with the BDI, while statistically significant, still suggests a practical level of discriminant validity between the two constructs. This suggested that depressed mood did not artificially inflate the association of these scales.

The psychometric properties of the MSS were based on an approximately 5:1 subject item ratio, which is within the 5-10:1 ratio rule of thumb for instrument development. Additionally, the obtained factor loadings above .60 on 21 of the 28 items suggested that the sample size was adequate (Guadagnoli & Velicer, 1988).

Despite the strengths of the MSS, there were some potential weaknesses with this study. The normative population used in this research was initial entry trainees. While this is a strength in terms of attention to a often ignored population, generalizability to other military personnel populations is limited. Local norms should be developed to reflect the racial and cultural make up of other geographical regions.

The subscale scores did not yield significantly different data than the overall score which suggests that there is not a unique multidimensional element to the types of stressors that individuals respond to in BCT.

Research using the MSS is currently in progress investigating the relation between stress, depression, anxiety, and outcome variables such as dietary selection, and dietary intake.

Additional studies investigating the performance of the MSS against other measures of adjustment or coping may be useful. Overall, it appears that the MSS offers an adequate assessment of stress that can allow military personnel to make useful programmatic changes and evaluate the role of the various basic training experiences on stress.

Acknowledgements

The research project was sponsored by the Department of the Army, Cooperative Agreement number DAMD 17-97-2-7013. The content of this paper does not necessarily reflect the position for the new policy of the government, and no official endorsement should be inferred.

The investigators would like to acknowledge the support of the military staff of Ft. Jackson, SC and the Training and Doctrinal Command of the Army. Special recognition is made for Chief Warrant Officer Azzalee Brown, SGM Gerald Butts, and SGM Whitehurst. Finally, the contributions of our research associates, Stephen Mayville, and Nicole Mickley, are recognized for their perseverance and dedication to the collection and analysis of the data.

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Table 1. Description of the study sample

		Week 1 (n=139)	Week 8 (n=92)
Age (yr)	M=	20.91	21.04
	SD=	3.19	3.39
Males		55.8%	58.7%
Females		44.2%	41.3%
Caucasian		55.0%	57.6%
African-American		26.4%	23.9%
Other Minority		18.6%	18.5%

Table 2: Mean and Standard Deviations of Perceptions of Stress

Item	M	SD
Lack of privacy	1.42	1.28
Lack of personal time	2.27	1.20
Feeling tired	2.28	1.38
Being yelled at or feeling verbally attacked	1.74	1.51
Being criticized	1.61	1.42
Lack of contact with family and friends	2.63	1.39
Limited time to eat	1.79	1.31
Limited food choices	0.88	1.19
Muscle cramps, soreness, blisters	1.68	1.46
Lack of sleep	2.41	1.44
Limited clothing options	0.76	1.25
Memorizing information	1.99	1.38
Classroom or phase testing requirements	1.79	1.44
Not knowing required information	2.17	1.41
Fear of doing something wrong	2.25	1.35
Being told what to do	1.23	1.37
Being embarrassed	1.61	1.49
Not understanding a command or regulation	1.81	1.37
Not being fast enough	1.96	1.37
Being graded on your performance	1.76	1.38
Decreased social life	1.68	1.53
Standing in formation	0.96	1.30
Completing rifle drills	0.78	1.23
Completing marches (e.g., sounding off/length)	0.92	1.26
Being publicly reprimanded or corrected	1.45	1.44
Being punished for someone else's mistakes	2.47	1.31
Failing a Physical Training test	2.39	1.43
Failing an inspection	2.10	1.43

Table 3

Factor Loadings

Item	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6

Subscale Reliability for final version of MSS

Scale (Number of Items)	Internal consistency (n = 139)	Item total ranges	Test-Retest (n= 93)
Personal Evaluation (5)	.90	.68-.80	.61
Personal Restriction (6)	.88	.63-.76	.60
Public Reprimand (5)	.91	.67-.82	.55
Regimentation (5)	.84	.58-.75	.51
Knowledge Deficits (4)	.90	.65-.81	.60
Physical Complaints (3)	.84	.72-.79	.63
MSS total (28)	.96		.65

Table 4

Correlations Between Subscale Totals, MSS-Total and WSI-Total

Subscale total	WSI Total	PSS Total	BDI Total	BAI Total
Personal Evaluation	**.52**	**.48**	**.36**	**.46**
Personal Restriction	**.59**	**.47**	**.37**	**.55**
Public Reprimand	**.61**	**.51**	**.36**	**.43**
Regimentation	**.45**	**.50**	**.39**	**.38**
Knowledge Deficits	**.61**	**.48**	**.39**	**.48**
Physical Complaints	**.57**	**.45**	**.32**	**.43**
MSS Total	**.67**	**.57**	**.44**	**.55**

all correlations were significant at $p < .0001$

TASK VI

Appendix C

Abstract that describes validation study for the Digital Photography Method

(submitted for presentation at NAASO, 2001)

Validation of digital photography and visual estimation methods for measuring food selections and food intake. *D.A. Williamson, R.Allen, A. Alfonso, P. Davis-Martin, S.B. Mayville, B. Hackes, and A.E. Hunt.* Pennington Biomedical Research Center and Louisiana Tech University

Valid measurement of food selections and food intake of humans has proven to be a difficult measurement problem. One method is direct observation of food selections and plate waste. The difference between these two variables provides an objective measure of food intake. This measurement approach is most applicable in institutional settings such as cafeterias. Traditionally, direct observation of food intake has been accomplished using a method called visual estimation. This approach requires human observers to categorize foods that are selected, and estimate portion sizes for each food before and after eating. One disadvantage of the visual estimation method is that it requires human observers to be present in the cafeteria at the time of food selections and return of uneaten foods, which is time consuming and somewhat intrusive and disruptive. An alternative method that was developed by this research team is called the digital photography method. This approach is similar to visual estimation, but instead of directly observing food selections and plate waste in the dining facility, digital video photographs are made of foods before and after eating. The digital photograph of each eating episode is evaluated by trained observers at a later time. Both methods require comparison to "food standards" and the data are converted to food servings, kilocalories, macronutrients, or grams of food using nutrient databases. The primary aims of this study were to: 1) test the validity of the visual estimation and digital photography methods against known food standards that had been carefully weighed and measured before and after eating, 2) test the association between the two estimation procedures, and 3) to evaluate interobserver agreement of portion sizes within the two methods. To accomplish these aims, 60 plates of food were prepared. For each plate, six observers (three for each estimation method) estimated portion sizes before and after eating. The results indicated that both estimation methods yielded results that were highly correlated with the food standards and that the agreement for portion sizes among the observers was very high. However, as portion size increased, both methods were biased toward underestimation of food selections and food intake. At the largest serving sizes, the visual estimation procedure was less biased in comparison to the digital photography method. These findings support the validity of these two methods for measuring food selections and food intake and indicate that both methods can be improved with certain types and amounts of food.
Funded by Dept of Defense contract, DAMD 17-97-2-7013

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TASK VI

Appendix D

Protocol for Study 2: Test of two interventions for modifying food selections

Enhancing Military Diets 2: Test of interventions to change eating habits of IET Students at Fort Jackson, SC

Principal Investigator
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PBRC Investigators
Pamela Davis Martin, Ph.D.
Ray Allen, Ph.D.

PBRC Medical Investigator
Donna Ryan, M.D.

Primary Aims

1. Test the effectiveness of two interventions for changing the food selections of soldiers in Basic Combat Training (BCT)
2. Measurement of food selections and food intake (with an emphasis on foods that are good sources of calcium, folate, and antioxidant nutrients) of soldiers in BCT at the beginning and end of the eight week BCT period.
3. Measure changes in body weight, body fat, and lean body mass over the eight-week period of BCT.
4. Measure indices of injury and attrition during BCT.
5. Assess dining service practices, e.g., availability of foods that are targets of the intervention, preparation of these foods, and presentation of these foods, to evaluate the implementation of a health promotion program in the dining facilities.
6. Assess implementation of the nutrition education intervention to be administered by Drill Sergeants.

Background Information

Many factors affect the health and performance of soldiers. One of these factors, healthy nutrition of soldiers, has been a primary concern of the military in recent years (Committee on Military Nutrition Research, 1993; 1995). One of these dietary concerns is adequate consumption of foods that are high in calcium, folate, and antioxidant nutrients, i.e., milk products, vegetables, fruit, and grains. In a recently completed study from our laboratory (Williamson, Allen, Martin, et al., 2000), we found that soldiers in BCT did not consume adequate amounts of these foods relative to the recommendations of the USDA for active adults. In this study, we found that soldiers generally consumed most (92% on average) of the foods that they selected in the serving line and that one could predict consumption very accurately based upon food selections ($r = .98$). Based upon this information, this study targets modifying food selections with the expectation that food intake will also be changed. This assumption will be tested, as a part of the study design.

In planning the design of this study, a video teleconference was conducted on July 25, 2000. During the two-hour video teleconference, the findings of the first study at Ft. Jackson were presented and discussed and the research design of the second study was developed. In attendance were: the investigative team from Pennington Biomedical Research Center, Alice Hunt, R.D., Ph.D. (external dietary consultant), LTC Vicky Thomas (representing the Office of the Surgeon General), COL Margaret Applewhite (San Antonio; Chief Army Dietitian for the Project), Harris Lieberman, Ph.D. (representing USARIEM), SGM Gerald Butts (representing TRADOC) and SGM Whitehurst and Mr. Don Davis (representing dining facilities management at Ft. Jackson, SC). Also, Dr. Williamson traveled to USARIEM on August 2, 2000 to brief Andrew Young, Ph.D. on the findings of the first study and plans for the second study. From these discussions, the final plan for the proposed study was developed. Both of these studies are funded as a part of contract DAMD 17-97-2-7013.

Significance of the study for the military

Consuming adequate amounts of calcium in the diet is important to assist in preventing injuries such as stress fractures and other skeletal and muscular problems (Committee on Military Nutrition Research, 1998). The best food source for calcium is dairy products. One goal of this study is to increase the milk selections of BCT soldiers to achieve the minimal USDA requirement (for active adults) of 3 or more servings of milk products per day.

Adequate intake of folate reduces the risk of birth defects called neural tube defects (Scott, Kirke, & Weir, 1990). In 1998 the U.S. Army Surgeon General implemented policy to increase folate intake. Previous research has found that 45% of BCT soldiers consume less than 60% of the recommended daily allowance for calcium and folate. The best food sources of folate are: fortified grains, legumes, fruits, and vegetables. One goal of this research project is to increase the selections of grains and vegetables by BCT soldiers to achieve the minimal requirement (for active adults) of at least 11grain servings per day and at least 5 vegetable servings per day.

Fruits and vegetables are excellent sources of antioxidant nutrients, minerals, fiber, and other important nutrients. Leading health organizations, i.e., the National Cancer Institute, the U.S. Department of Health and Human Services (DHHS), the U.S. Department of Agriculture (USDA), and the National Academy of Sciences, have urged all Americans to eat 5 or more servings of fruits and vegetables daily. For acitive adults, the USDA recommends eating at least four servings of fruit per day. All of these expert committees agree that eating more fruits and vegetables may reduce the risk of developing cancer and other chronic diseases. Based upon these considerations, one goal of this study is for BCT soldiers to select and consume at least four servings of fruit per day.

It should be noted that the Committee on Military Nutrition Research recommends that emphasis be placed on meeting the recommendations of the Dietary Guidelines for Americans (USAD/DHHS, 1995), consuming these nutrients from food sources, rather

than supplementing with individual nutrients (Institute of Medicine, 1999). This study is the second in a series of investigations by the Pennington Biomedical Research Center to address the question of how the military can improve the healthy nutritional intake of soldiers in BCT. TRADOC dining facilities for soldiers in BCT (also called IET students) provide an excellent setting to test various strategies to improve healthy nutrition. Soldiers in BCT eat most meals in the dining facilities and are forbidden to eat foods outside the military environment. Therefore, "contamination" of total food intake (per day) from external food services is minimal for these soldiers. This study will be conducted at Fort Jackson in Columbia, SC with two Brigades (1st Brigade - 3-13th IN and 4th Brigade - 1-61st IN). The two Brigades dine in two separate dining facilities. One is a relatively new "Starship" facility and the other is an older "Rolling Pin" facility. During recent visits to Fort Jackson, the military leadership and the dining facility contract personnel provided full support for the research project.

Research Design

The study sample will be the soldiers in BCT from four companies (two from 3-13 IN and two from 1-61 IN). There will be approximately 250 solders in each of the four companies. Therefore, the maximum number of volunteers would be 1,000 soldiers. We anticipate that about half of the 250 soldiers in each company (n = 125) will volunteer to participate in the study. Therefore, the study sample is anticipated to be approximately 500 soldiers and will be no greater than 1,000 soldiers (if every soldier in the four companies volunteered to be in the study). The soldiers from each brigade will eat in two different dining facilities. Two interventions to modify the food selections of soldiers will be tested/evaluated in this study. One intervention, a nutrition information program, will involve providing information (in the dining facilities) about the importance of selecting fruits, vegetables, grains, and milk products and will prompt the selection of these foods on the serving line. This nutrition information program will be implemented for all soldiers in the two dining facilities. The second intervention will include this nutrition education program and will also include a nutrition education program that will be administered by the Drill Sergeants. This program will be implemented in only the two companies of one brigade (either 3-13 IN or 1-61 IN, selected by chance), and all soldiers in the two companies of this brigade will receive this nutrition education program. The research volunteers from the four companies will be evaluated as a part of this program evaluation study. Only those soldiers who agree to participate (via written consent) in the evaluation study will be research participants. Volunteers from the four companies will sign consent forms to inform them of the study and all soldiers will be allowed nonparticipation and will be allowed to withdraw from the study without penalty at anytime during the study. The research design will involve collection of data from two dining facilities at two points in time (beginning and end of BCT). With the exception of demographic data (to be collected at the beginning of BCT) and reports of injury and attrition (to be collected at the end of BCT), the same measures will be collected at each measurement point. As described below, the assessment procedures will measure: 1) food selections and plate waste, 2) body weight and height, 3) body composition - percent body fat and percent lean mass, 4) behavioral factors - dietary restraint, overeating habits,

perceived hunger, and food cravings, 5) data related to the implementation of the two interventions that will be evaluated in the study.

Methods

Research Participants

The study sample will be selected from approximately 1,000 male and female BCT soldiers between the ages of 18 and 35 years. Soldiers who are younger than 18 or older than 35 will be excluded from the study. These participants will be identified from four companies (of approximately 250 soldiers per company) from two Brigades at Fort Jackson (1-61 IN and 3-13 IN) that were selected for study by TRADOC. Neither the social security numbers nor the names of the participants will be entered into the database. Therefore the identities of the participants will be unknown to the research team. This anonymity will be achieved using the bar codes of each soldier's identification card. The details of this encrypted code are described below. Participants will sign consent forms at the orientation period of BCT. They may refuse to participate and can withdraw from the study at any time. A copy of the informed consent form that is to be read and signed by volunteers is attached. To participate in the study, participants from this sample must read and sign the attached consent form. There will be one witness of this consent procedure and the witness and the Principal Investigator will each sign the consent forms. Military personnel will be briefed about the rights of research participants in a meeting that will precede the initiation of the study. This briefing will emphasize the rights of research participants, including the right to refuse participation and the right to withdraw from the study, without penalty. The briefing will also include a video tape presentation of the procedures that will be used in the study. We plan to minimize the role of Drill Instructors or other military leaders in this briefing. We will request that they leave the room while we discuss the rights of human subjects and solicit research participants. We will invite an ombudsman who is not connected with the study to be present during the entire presentation and process of soliciting volunteers and obtaining consent to participate in research. If during the course of the study, a volunteer decides to discontinue participation in the study, the person may do so without penalty. The investigators will diligently monitor any actions against such persons should they decide to withdraw from the study or refuse to participate from the beginning of the study. In a similar study that has recently been completed, we recruited soldiers from these same brigades at Ft. Jackson. Using the methods described above, approximately 45% of the eligible soldiers agreed to volunteer for the study. This rate of participation and the continued participation of most soldiers in the study, suggests that this procedure is not overtly coercive and that it was successful in protecting the rights of the volunteers to refuse participation.

Power Analysis

The sample size for the study was determined by the power analysis conducted by the study statistician, Anthony Alfonso. The details of the power analysis can be found in Appendix 1. The power calculations were based upon the findings from the initial study conducted at Ft. Jackson, which used the digital photography method for measuring food selections and food intake. For the purpose of this analysis, three food categories were selected as the primary targets of the intervention: fruit, milk products, and vegetables. The data were represented in two ways: 1) mean servings of food consumed and 2) the percentage of soldiers that met the recommended (RDA) number of servings (as defined by the Food Guide Pyramid). Three assumptions were made: 1) an attrition rate of 25% could be expected, 2) the experimental group and the control group would differ by one serving at the eighth week of BCT and 3) at the end of BCT, there would be improvement (meeting RDAs for all three food categories) by 15% of the experimental group in comparison to the control group. From these assumptions, the power analysis led to the estimate that between 90 and 250 soldiers should be recruited for each group (experimental and control). In the first study, approximately half of the soldiers volunteered for the study at the beginning of BCT. Using this experience, we estimated that by starting with a sample of approximately 1,000 soldiers, we should be able to recruit about 500 volunteers, i.e., 250 participants per group, i.e., experimental and control.

Bar Code Methodology

Soldiers in BCT will be studied at the beginning (first week) of the eight-week BCT course and again at the completion (eighth week) of BCT. Correlation of the data requires the ability to identify all data collected from an individual subject. However, it is desirable to keep the subject's identity anonymous in order to protect the privacy of the volunteers. The use of bar code technology allows both of these goals to be attained. This will be accomplished using notebook computers, bar code scanners, and palm computers. At no time will data be transferred via the internet. All data transfer will be accomplished using magnetic media within the PBRC local area network. Each soldier is issued (by the Army) an identification card with identification information encoded on the back of the card in a bar code. The information contained in the bar code is encoded and unique to the soldier. The encoding prevents the research team from determining the soldier's Social Security Number or any other personal information about the soldier. By using this unique identifier, the data from various sources can be linked to an individual without knowing the identity of an individual soldier. Therefore, the names or social security numbers of the soldiers <u>will not</u> be collected at any time during the study. Furthermore, the ID numbers of soldier's will not be a part of the data set for statistical analyses. These unique identifiers will only be used to link various pieces of data over time to an individual soldier. This methodology will allow the identity of all participants to remain anonymous.

Interventions

The name that we have selected for this program is "Power Up." The name was selected to convey the message that healthy nutrition could enhance performance and endurance.

The Power Up program includes two components that will be used to modify the food selections of soldiers: 1) nutrition information and 2) nutrition information and nutrition education. One of these interventions will be implemented in one of the dining facilities and the other will be implemented in the other dining facility. The assignment of an intervention to a particular dining facility will be determined randomly prior to the initiation of the study. It is important to note that the nutrition information intervention has been approved by the commanding officers and will be implemented for all soldiers in the two dining facilities. Also, the nutrition education intervention will be implemented for all soldiers in the two companies that are assigned to this condition. Therefore, these programs are a part of BCT for soldiers in these brigades/companies. The research volunteers are only those soldiers who agree to participate in the assessment procedures that are described below.

Nutrition information. In our previous research project, we found that BCT soldiers wanted to learn about healthy eating habits, but did not use nutrition information in the dining facilities to make informed decisions about food selections. To increase selections of fruits, vegetables, grains, and milk products, a focused program to target the selection of these foods will be developed and implemented in both dining facilities. The materials in Appendix 2 illustrate the design of the health promotion items that will be placed in both dining facilities. This intervention will include the following elements: 1) focus stresses fruits, vegetables, grains, and dairy products, 2) strong visual aids, repetition, and consistency of the nutrition information program, emphasizing a graphic design that will be colorful and bold, 3) use of Food Labels to identify those foods that should be selected to achieve improved intake of targeted nutrients, 4) a visually salient Menu Board that shows the foods that will be served that day and the foods that are targets for selection, 5) Motivational Buttons worn by Servers that will remind soldiers to select targeted foods, and 6) enhancement of the availability of foods that are easily consumed and are good sources of calcium, folate, and antioxidant nutrients.

Nutritional Education. To implement this program, a course of instruction to be taught by the Drill Sergeants has been developed. The entire course can be taught in 1.5 hours and it has three modules that last about 1/2 hour each. This modular approach allows for flexibility in the actual time that is allotted to this instruction in a given class. BCT soldiers will receive training using the Power Up program during the first week of BCT. This training will be integrated into the course material on health and fitness. A training manual has also been developed for the drill sergeants to follow when teaching their students. These materials can be found in Appendix 3. The nutrition education provided to BCT soldiers will be integrated into the existing curriculum of BCT, which includes several sessions related to health and nutrition. Portable (pocket-size) reference manuals have been developed for the Drill Sergeants and for BCT soldiers so that the lessons can be studied in the barracks, in the classroom, and can be easily carried in the pockets of the soldier's uniform. Also, overheads and Power Point slides have been developed for use in teaching the course (to the BCT soldiers and to drill sergeants).

A teaching manual to train the Drill Sergeants to teach the course has also been developed. Dr. Williamson and other members of the research team will train the drill

sergeants that are assigned to the companies who will be provided the nutrition education. The Nutrition Education course for the Drill Sergeants will be conducted approximately one to three days prior to the beginning of BCT. After completion of this study, these teaching materials will be available for training other drill sergeants, in the event that this approach is adopted as a part of BCT. The manuals that will be used for these purposes can be found in Appendix 3.

Format and content for the three training modules is described below:

Module One: Awareness (30 minutes)

In this module, soldiers will view a video-tape (produced by ComTel Productions, Inc for USARIEM and the U.S. Armed Forces) about healthy eating for soldiers and they will learn the reasons for increasing the consumption of fruits, vegetables, grains, and dairy products. The four components of this module are described below.

A. View a video-tape developed by USARIEM, called the Power Performance video. Stress performance, strength, and endurance as the reasons to select and consume more fruits, vegetables, grains, and dairy products.
B. Introduce the findings from previous research: BCT soldiers do not consume adequate amounts of fruits, vegetables, grains, and milk products.
C. Explanation about inadequate consumption of these foods and the effects upon performance, strength, and endurance.
D. Introduction of the Power Up program

Module Two: Power Food Focus (30 minutes)

In this module, soldiers will learn about the Power Up program and the foods that are served in the dining facilities that have been targeted for increased consumption. Overheads that illustrate food trays with "good" versus "poor" choices for each of the targeted food groups are provided. The training manual explains why these are either "good" or "poor" choices. The portable reference manuals will be provided to each soldier (and drill sergeant) during this section of the course. Also, examples of food trays with "good" versus "poor" representation of fruits, vegetables, grains, and milk products will be provided. Finally, the POWER UP acronym will be explained: P = Protein, O = orange (vegetables and fruits), W = Winter vegetables, E = Extra Dairy, and R = Raw Vegetables. For each food group, there will be discussion, as outlined below:

A. Brief overview of the Food Guide Pyramid, stressing areas of specific concern – namely, fruits, vegetables, grains, and dairy products.
B. Fruits. Closer look at fruits and their benefits. What food sources are good selections of fruit? Discuss the details in the hand reference manual.
C. Vegetables and grains. Differences in vegetables from green leafy to yellow to potatoes. Discussion of the details about vegetables and grains in reference manual.
D. Dairy and Calcium. Focus on the role of dairy products and healthy nutrition. Describe myths of digestion. Explain Lactose intolerance, which has special relevance to African-American soldiers. Stress

importance and other food sources for calcium. Discussion of the details about dairy products in reference manual.

Module Three: Six exercises to test for comprehension and understanding

In this final module a set of tests have been developed to help students determine healthy versus unhealthy food selections.

A. In these six exercises, overheads showing food trays with different varieties of foods will be displayed. Soldiers will be asked questions to help them recognize Power Up foods and to stimulate discussion in classes.
B. Portable reference manuals will be reviewed for a second time. Repetition of training material will be emphasized, using the reference manuals as a reminder of key guiding principles of the POWER Up program.
C. With the drill sergeants, the trainers will stress the use of positive feedback to reinforce healthy food selections as opposed to punishment of unhealthy food selections. Appropriate strategies for prompting the selection of fruits, vegetables, grains, and dairy products will be discussed. Also, appropriate methods for giving verbal recognition to individuals and the entire company for making these food selections will be discussed.

Drill Sergeants will be instructed to recognize the selections of Power Up foods by BCT soldiers and to provide social reinforcement (e.g., verbal praise) for making these food selections. Soldiers who choose Power Up foods will not be given special privileges or rewards, however. During health and nutrition classes, Drill Sergeants will ask questions related to the content of the Power Up portable manuals in order to reinforce soldiers who have learned the material. During meals, utilization of the Power Up program will not require additional time for BCT soldiers. The menu boards and other educational or health promotional materials will be designed to guide food selections without any additional studying or other time requirements.

Schedule of Data Collection

Data will be gathered during two seven-day periods at the beginning and end of BCT for each company. Two days during each seven-day period will be required for the collection of data in the two dining facilities. At each dining facility, measures of food selections and food intake will be gathered at all three meals on two consecutive days. Since the BCT training schedule for the two brigades will start at different times, all outcome measures will be collected on different days for the two brigade/dining facilities. Menus across the two dining facilities will be matched as closely as possible so that the same foods will be served during each two-day period for each facility. Therefore, a total of eight days (two two-day periods at the beginning and end of BCT) will be required for this data collection. Survey data and measures of body weight, height, and body composition will be collected during the two-day measurement periods. Prior to the

collection of data the soldiers will be briefed about the study procedures and their questions will be answered. The measures and methods for data collection are described in greater detail in the following sections.

Measurement of food selections and plate waste using digital photography

Methods that have been used on previous Army studies to determine consumption of food, such as Visual Estimation, are impractical in this study due to the intrusiveness of the conventional visual estimation procedure. A novel methodology will be used to determine foods selected by soldiers and to estimate foods consumed by plate waste. This procedure is called digital photography of food selections. We tested this procedure in a recent study at Ft. Jackson and found it to be a reliable and unobtrusive method for measuring food selections and food intake. The procedures for gathering digital photographs and converting them to Food Guide Pyramid categories/servings are described below.

A digital video camera mounted on a tripod will be used to photograph the soldier's tray as they arrive from the serving line to the dining area. The camera angle is such that only objects that are placed on a table beneath the camera are recorded. Therefore, a soldier's face or any other identifying information will not be recorded. The time requirements for this procedure are minimal. The digital video camera takes 30 frames per second. The total time for recording one tray is about 1 to 5 seconds. This process does not significantly influence the normal routine of soldiers as they move from the serving line to being seated and from being seated to entering the tray return area.

The photographs will include the beverage(s) selected by the soldiers. A card containing the bar code, which will be given to the soldier at the start of the serving line, will also be photographed along with the tray. By coupling the bar code and the soldier's ID bar code, this procedure enables the digital photographs to be linked to an individual soldier across many different measurements over time. The digital video camera has the capability to capture still images and record them to a 60-minute tape eliminating the need to change storage media during data collection. Plate waste data will also be collected using digital photography by capturing pictures of the trays after each soldier has finished eating. Food consumption can be estimated by subtracting the plate waste data from the food selection (before eating) data. The details of this process are provided below.

To convert the digital photographs to Food Guide Pyramid servings, data collected with the video camera will be analyzed upon return to PBRC. A Desktop computer with the still image capture card installed will be used to capture a digital image of each tray that has been photographed. A custom designed computer application will allow images of the tray before food consumption and following food consumption (plate waste) to be displayed side-by-side on the computer screen. The software will also have data fields to enter amounts of foods selected and consumed. Serving sizes will be estimated using standards for each food that will be established prior to the meal. The entered data will be stored in a database along with the number assigned to the soldier. Analysis of the

images and entry of the data will be performed by a trained dietitian. In the initial test of these procedures, estimates of interrater reliability found that the classification of foods and judgements of portion sizes were made with satisfactory accuracy/agreement by the three dietitians that will be used in this study.

Classification of Food Selections

The food selections, as determined from the digital photographs, will be analyzed using the USDA food grouping system of the Food Guide Pyramid (The Food Guide Pyramid. Washington, DC: US Dept of Agriculture; 1992. Home and Garden Bulletin No. 252.). This method of classification will provide data of the food group selections of each soldier.

Assessment of plate waste and estimates of foods consumed

Prior to consumption and at the end of the meal, serving plates will be digitally photographed, as described above. The amount of food consumed will be estimated by subtracting plate waste (food left) from the food selected. The calculation of foods intake is made for each individual food that is selected by a soldier. The food intake is then summarized in terms of Food Guide Pyramid categories. Also, estimates of caloric intake can be derived from these data.

Assessment of the Purchasing, Cooking, and Delivery Records

In order to accurately specify the food groups for each food, the following methods will be used on the day that food selections are tested. Research staff will collect the purchasing and delivery records of items that have been ordered for the dining facility. Also, the menu for that day will be acquired. During food preparation, research staff will record, in recipe-style format, how food items were prepared and served. This information will be used to insure that each food served is classified into the most appropriate food category.

Measurement of Body Weight, Questionnaires, and Injuries

During each two-day measurement period, body weight, height, and body composition will be measured in a one-hour group assessment session. Body weight will be measured in standard military clothing using a body scale that also measures body composition via body impedance assessment. This device, a Tanita body composition analyzer measures body weight and body composition in one step. The soldier simply steps on the scale in bare feet and both variables are measured. Height will be recorded from the participant's ID card during the initial (week 1) assessment period. Body composition will be measured using body impedance assessment, a convenient, noninvasive method that has been validated in studies of body composition (Heymsfield, et al., 1998). Body impedance involves passing a very low electric current (that is safe and not noticeable to the person) through a person's body. The electric current passes more easily through lean

muscle than fat tissue. Using the resistance of this electrical current, in combination with height, weight, and gender, a person's fat mass, lean mass, and hydration level can be validly estimated. This procedure has been validated in many studies (Heymsfield, et al., 1998). The methodology of this study will involve using a single device to measure body weight and body impedance. These data will be automatically captured by a computer so that there will be no need to manually record the data (which saves time). The measurement device is a Tanita body composition analyzer (model TBF-310). This body composition analyzer has met the standards of safety for a Class B digital device, pursuant to Part 15 of the Federal Communications Commission rules. The only restriction in its use is that persons with a cardiac pacemaker should not use the device. It is not anticipated that this will pose a problem for this sample (the consent form will note this caution, in case there is a person with this implanted medical device).

During these sessions, soldiers will be administered two questionnaires about eating habits: 1) the Eating Inventory, which assesses intent to diet, overeating habits, and perceived hunger (Stunkard & Messick, 1985; and 2) the Food Craving Inventory, which measures craving of specific foods, e.g., sweets, starches, and dietary fat (White, et al., 1999). Both of these questionnaires are self-administered and have been demonstrated to be reliable and valid. There will be a unique bar code on each questionnaire. This bar code will be linked to each individual soldier by coupling the barcode on the questionnaires with the unique bar code on the ID card of each soldier. Volunteers from each company will meet as a group, in a classroom where the questionnaire and weight and body impedance data will be collected. Total time for collecting these data will be one hour in the first week of BCT and one hour during the eight week of BCT.

During the first week of BCT one very short questionnaire (requiring less than 5 minutes) will also be administered. This survey, called the Demographics Questionnaire, will gather information about age, gender, and race/ethnicity, dietary restrictions, and intent to diet to lose weight during BCT. This questionnaire will not be administered at Week 8. Volunteers can refuse to answer any questions on the survey instruments without penalty and can continue to participate in the research project without penalty, if they refuse to answer any question. At the end of BCT, we will collect information on number of sick calls (as an index of injuries) for each company. The questionnaires can be found in Appendices 4, 5, and 6.

Assessment of Implementation of the Interventions

One 50% FTE employee will monitor the implementation of the nutrition information and nutrition education programs throughout the eight weeks of BCT for the study sample. The study monitor will record the presence versus absence of the various elements of the two programs on a daily basis. This information will be summarized each week and will be sent via email to the Principal Investigator. If significant deviations from the study protocol occur, the Principal Investigator will notify appropriate staff at Ft. Jackson so that these problems can be addressed quickly.

Statistical Methods

Correlational procedures such as Pearson Product Moment Correlations, Chi Square, and multiple regression analysis will be used to test the association among variables at each assessment point and across the longitudinal study. Procedures such as analysis of variance will be used to test for changes in behavior, body weight, body fat, and lean body mass over the eight weeks of BCT as a function of the two interventions.

Duties and Responsibilities

Donald Williamson, Ph.D. is the Principal Investigator for this research project and he will supervise and coordinate all aspects of the study. Pam Davis Martin, Ph.D. will develop and implement the psychological and behavioral aspects of the study. Ray Allen, Ph.D. will develop and supervise the implementation of the information technology that is required by this study. Donna Ryan, M.D. is the medical investigator for all of the studies conducted for the Department of Defense by the Pennington Center. Her responsibilities involve oversight of the projects and reporting of research activities to DOD on a quarterly and annual schedule.

Adverse Events

Adverse experiences that are both serious and unexpected will be immediately reported by telephone to the USAMRC Office of Regulatory Compliance and Quality (301-619-2165) during duty hours. During nonduty hours, adverse events will be reported by calling 301-619-2165 and by sending information by facsimile to 301-619-7803/7893). A written report will follow the initial telephone call within 3 working days. The report will be addressed to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

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Items in the appendices

1. Power analysis for determination of sample size
2. Power Up materials for the health information program in the dining facilities
3. Power Up manuals for training drill sergeants and for BCT soldiers
4. Demographics Questionnaire
5. Eating Inventory
6. Food Craving Inventory

Appendix 1. Power analysis for determination of sample size

The primary interest of this study is the effect of diet modification on military subjects in basic combat training (BCT). The design proposes two groups a control and a treatment group. In the first study, Enhancing Military Diet I, information on nutritional consumption was collected and analyzed. From this analysis it was discovered that the percent of soldiers meeting the USDA recommended daily allowances (RDAs) for fruit, milk and vegetable consumption was low. Since these foods contribute to the intake of calcium, folate acid and antioxidants, which was of interest to the first study, the power analysis for the second study is based on this information.

Using mean consumption and proportion meeting the RDAs food categories, estimates from the data collected in the eighth week of BCT, power analyses for fruit, milk and vegetables were performed. Two-sample T-test analyses were done to calculate the sample size needed to detect a one serving difference in means for fruit, milk and vegetable consumption. Two proportions power analyses were performed to calculate the sample size needed to detect a 15% difference in soldiers meeting the RDAs.

The tables 1 and 2 below present the results of the analyses. The two sample T-test sample size was calculated under a type I error rate of 5%, two-tailed alternative and standard deviation (S.D.) was equal for both groups. The two proportions sample size was calculated under a type I error rate of 5% and a two-tailed alternative.

Table 1. Power results for Mean Consumption.

Food Consumption	S. D.	Power	n	Mean Difference
Fruit	1.18	.8025	92	.5
		.8014	40	.75
		.8025	23	1.0
Milk	2.01	.8005	265	.5
		.8009	118	.75
		.8036	67	1.0
Vegetable	1.96	.8013	253	.5
		.8029	113	.75
		.8045	63	1.0

Table 2. Power results for Proportion meeting RDAs.

Food Consumption	Eighth Week Proportion	Power	n	% Difference in Proportions
Fruit	0.05	.8008	153	10
		.8011	82	15
		.8043	54	20
Milk	0.495	.8001	407	10
		.8009	183	15
		.8038	103	20
Vegetable	0.525	.8003	427	10
		.8021	187	15
		.8027	103	20

Assuming a mean difference of one serving or a 15% difference between proportions is to be detected with power of at least 80% between the two groups for any one food, the end of study sample size should be 67 and 187 per group, respectively for the two types of analyses. From the first study, a dropout rate of 25% was observed. Taking this assumption into account, the appropriate sample sizes to maintain a power of at least 80% should be 90 and 250 per group respectively.

Appendix 2

Power Up materials to be displayed in the dining facilities to provide nutritional information to promote the selection of fruits, vegetables, grains, and milk products. Using these logos, a daily menu board will be constructed. This menu board is not depicted due to size of the board. Also servers will wear the Power Up buttons to remind soldiers to select the proper Power Up foods, that will be labeled on the serving line.

Appendix 3

Power Up manuals to be used in training the Drill Sergeants and manuals that will be provided to BCT soldiers to help them learn about the value of eating fruits, vegetables, grains, and milk products.

Appendix 4

Copy of the Demographics Questionnaire

Enhancing Military Diets II

Demographics Questionnaire

SEX

Male	▲
Female	▲

AGE

0	▲	▲
1	▲	▲
2	▲	▲
3	▲	▲
4		▲
5		▲
6		▲
7		▲
8		▲
9		▲

What is your ethnic origin?

- ▲ Caucasian
- ▲ African American
- ▲ Asian
- ▲ Hispanic/Mexican American
- ▲ American Indian
- ▲ Other _____

Dietary Restriction (mark all that apply)

- ▲ Vegetarian
- ▲ Lactose Intolerant
- ▲ Caloric Restriction

What is your current status? (choose one)

Active Duty	▲
Army Reserve	▲
National Guard	▲

Appendix 5

Copy of the Eating Inventory

Appendix 5

Enhancing Military Diets II

Eating Inventory

For items 1-36, please answer either "True" or "False".

	TRUE	FALSE
1. When I smell a sizzling steak or see a juicy piece of meat, I find it very difficult to keep from eating, even if I have just finished a meal.	▲	▲
2. I usually eat too much at social occasions, like parties and picnics.	▲	▲
3. I am usually so hungry that I eat more than three times a day.	▲	▲
4. When I have eaten my quota of calories, I am usually good about not eating anymore.	▲	▲
5. Dieting is so hard for me because I just get too hungry.	▲	▲
6. I deliberately take small helpings as a means of controlling my weight.	▲	▲
7. Sometimes things just taste so good that I keep on eating even when I am no longer hungry.	▲	▲
8. Since I am often hungry, I sometimes wish that while I am eating, an expert would tell me that I have had enough or that I can have something more to eat.	▲	▲
9. When I feel anxious, I find myself eating.	▲	▲
10. Life is too short to worry about dieting.	▲	▲
11. Since my weight goes up and down, I have gone on reducing diets more than once.	▲	▲
12. I often feel so hungry that I just have to eat something	▲	▲
13. When I am with someone who is overeating, I usually overeat too.	▲	▲
14. I have a pretty good idea of the number of calories in common food.	▲	▲
15. Sometimes when I start eating, I just can't seem to stop.	▲	▲

16. It is not difficult for me to leave something on my plate. ▲ ▲

17. At certain times of the day, I get hungry because I have gotten used to eating then. ▲ ▲

18. While on a diet, if I eat food that is not allowed, I consciously eat less for a period of time to make up for it. ▲ ▲

19. Being with someone who is eating often makes me hungry to eat also. ▲ ▲

20. When I feel blue, I often overeat. ▲ ▲

21. I enjoy eating too much to spoil it by counting calories or watching my weight. ▲ ▲

22. When I see a real delicacy, I often get so hungry that I have to eat right away. ▲ ▲

23. I often stop eating when I am not really full as a conscious means of limiting the amount I eat. ▲ ▲

24. I get so hungry that my stomach often seems like a bottomless pit. ▲ ▲

25. My weight has hardly changed at all in the last ten years. ▲ ▲

26. I am always hungry so it is hard for me to stop eating before I finish the food on my plate. ▲ ▲

27. When I feel lonely, I console myself by eating. ▲ ▲

28. I consciously hold back at meals in order not to gain weight. ▲ ▲

29. I sometimes get very hungry late in the evening or at night. ▲ ▲

30. I eat anything I want, any time I want. ▲ ▲

31. Without even thinking about it, I take a long time to eat. ▲ ▲

32. I count calories as a conscious means of controlling my weight. ▲ ▲

33. I do not eat some foods because they make me fat. ▲ ▲

34. I am always hungry enough to eat at any time. ▲ ▲

35. I pay a great deal of attention to changes in my figure. ▲ ▲

36. While on a diet, if I eat a food that is not allowed, I often splurge and eat other high calorie foods. ▲ ▲

Please answer the following questions by filling in the circle on your answer sheet corresponding to the letter of the response that is appropriate to you.

37. How often are you dieting in a conscious effort to control your weight?

▲ ▲ ▲ ▲

rarely sometimes usually always

38. Would a weight fluctuation of 5 lbs. affect the way you live your life?

▲ ▲ ▲ ▲

rarely sometimes usually always

39. How often do you feel hungry?

▲ ▲ ▲ ▲

rarely sometimes usually always

40. Do your feelings of guilt about overeating help you to control your food intake?

▲ ▲ ▲ ▲

rarely sometimes usually always

41. How difficult would it be for you to stop eating halfway through dinner and not eat for the next four hours?

▲ ▲ ▲ ▲

easy slightly difficult moderately difficult very difficult

42. How conscious are you of what you are eating?

not at all slightly moderately extremely

43. How frequently do you avoid "stocking up" on tempting foods?

almost never seldom usually almost always

44. How likely are you to shop for low calorie foods?

unlikely slightly likely moderately likely very likely

45. Do you eat sensibly in front of others and splurge alone?

never rarely often always

46. How likely are you to consciously eat slowly in order to cut down on how much you eat?

unlikely slightly likely moderately likely very likely

47. How frequently do you skip dessert because you are no longer hungry?

almost never seldom at least once a week almost every day

48. How likely are you to consciously eat less than you want?

unlikely slightly likely moderately likely very likely

49. Do you go on eating binges though you are not hungry?

never rarely sometimes at least once a week

50. To what extent does this statement describe your eating behavior? "I start dieting in the morning, but because of any number of things that happen during the day, by evening I have given up and eat what I want, promising myself to start dieting again tomorrow."

not like me	little like me	pretty good description of me	describes me perfectly

51. On a scale of 1 to 6, where 1 means no restraint in eating (eating whatever you want, whenever you want it) and 6 means total restraint (constantly limiting food intake and never "giving in"), what number would you give yourself?

1 = eat whatever you want, whenever you want it

2 = usually eat whatever you want, whenever you want it

3 = often eat whatever you want, whenever you want it

4 = often limit food intake, but often "give in"

Appendix 6. Copy of the Food Craving Inventory

Appendix 6

Enhancing Military Diets II

FOOD CRAVING INVENTORY

A craving is defined as an intense desire to consume a particular food (or food type) that is difficult to resist.

Over the past month, how often have you experienced a craving for the food?

	1 Never	*2* Rarely (once or twice)	*3* Sometimes	*4* Often	*5* Always/ Almost every day
Cake	▲	▲	▲	▲	▲
Pizza	▲	▲	▲	▲	▲
Fried Chicken	▲	▲	▲	▲	▲
Gravy	▲	▲	▲	▲	▲
Sandwich Bread	▲	▲	▲	▲	▲
Sausage	▲	▲	▲	▲	▲
French fries	▲	▲	▲	▲	▲
Cinnamon Rolls	▲	▲	▲	▲	▲
Rice	▲	▲	▲	▲	▲
Hot dog	▲	▲	▲	▲	▲
Hamburger	▲	▲	▲	▲	▲
Biscuits	▲	▲	▲	▲	▲
Ice cream	▲	▲	▲	▲	▲
Pasta	▲	▲	▲	▲	▲
Fried fish	▲	▲	▲	▲	▲
Cookies	▲	▲	▲	▲	▲
Chocolate	▲	▲	▲	▲	▲
Pancakes or waffles	▲	▲	▲	▲	▲
Corn bread	▲	▲	▲	▲	▲
Chips	▲	▲	▲	▲	▲
Rolls	▲	▲	▲	▲	▲
Cereal	▲	▲	▲	▲	▲
Donuts	▲	▲	▲	▲	▲
Candy	▲	▲	▲	▲	▲
Brownies	▲	▲	▲	▲	▲
Bacon	▲	▲	▲	▲	▲
Steak	▲	▲	▲	▲	▲
Baked potato	▲	▲	▲	▲	▲

TASK VII

STRESS, NUTRITION AND IMMUNE FUNCTION LABORATORY

NONE

TASK VIII

METABOLIC UNIT PROJECT

NONE